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Issued April 1, 1910.

U. S. DEPARTMENT OF AGRICULTURE,
BUREAU OF ANIMAL INDUSTRY.—BULLETIN 120.
A. D. MELVIN, CHIEF OF BUREAU.

THE INTRACELLULAR ENZYMS OF PENICILLIUM AND ASPERGILLUS,

With Special Reference to Those of Penicillium Camemberti.

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WASHINGTON:
GOVERNMENT PRINTING OFFICE.
1910.

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LETTER OF TRANSMITTAL.

U. S. Department of Agriculture,
Bureau of Animal Industry,
Washington, D. C., October 9, 1909.

Sir: I have the honor to transmit herewith for publication a manuscript entitled "The Intracellular Enzyms of *Penicillium* and *Aspergillus*, with Special Reference to those of *Penicillium camemberti*," by Arthur W. Dox, chemist in cheese investigations in the Dairy Division of this Bureau. The experiments recorded in this paper have grown out of the work in connection with European varieties of soft cheese, which has been in progress for several years at Storrs, Conn., in cooperation between the Storrs Experiment Sation and this Bureau. Much of the work referred to has been concerned with the manufacture of the Camembert type of cheese, and the characteristic mold of this cheese, *Penicillium camemberti*, was the organism used by Doctor Dox in the enzym experiments described herein.

I recommend the publication of the article in the bulletin series of this Bureau.

Respectfully,

A. D. Melvin,
Chief of Bureau.

Hon. James Wilson,
Secretary of Agriculture.

CONTENTS.

Page.
Introductory 7
  Intracellular and extracellular enzyms 8
  Molds in general 9
  Penicillium and *Aspergillus* 9
    Distribution and economic importance 10
    Adaptability to artificial culture 11
    Sources of carbon and nitrogen 11
    Changes in the culture medium 13
    Separation of racemic mixtures 14
    Behavior toward antiseptics 15
Review of previously published work on the enzyms of *Penicillium* and *Aspergillus* 16
  The various methods of preparing the enzyms 16
  The particular enzyms found 17
  Summary of the literature 35
Experimental work 35
  Chemical differences in species of *Penicillium* 35
  The organism selected, *Penicillium camemberti* 36
  Choice of a culture medium 37
  Methods of culture 38
  Preparation of enzym powder 38
  Composition of enzym powder 38
  Experimental methods 39
  Enzym studies 39
Summary 64
Acknowledgment 65
Bibliography 66

THE INTRACELLULAR ENZYMS OF PENICILLIUM AND ASPERGILLUS, WITH SPECIAL REFERENCE TO THOSE OF PENICILLIUM CAMEMBERTI.

INTRODUCTORY.

The importance of the lower plants in their relations to the various biochemical processes constantly going on about us is being recognized more and more as new data are accumulated. This field of research, which was first brought to the notice of scientists some fifty years ago, has proved one of the most interesting in the whole realm of biological chemistry. Although at the present time we are only on the threshold of any adequate conception of the various chemical processes which constitute life in its lowest forms, sufficient knowledge has already been gained to form a working hypothesis, which not only explains many of the well-known phenomena but also aids in the discovery of new truth. Perhaps the most striking example of this fact is to be found in the development of our knowledge of enzyms or unorganized ferments, especially the intracellular enzyms. Most of the processes upon which life and growth are so intimately dependent can now be regarded as more or less simple chemical reactions, in which an enzym plays the part of a catalytic agent. Duclaux even goes so far as to suggest that the life of bacteria is probably nothing more nor less than the sum total of all the activities of the intracellular enzyms present.

Long after enzyms were known and studied in their relation to the digestion of food, chemical reactions of a similar nature which took place through the intervention of micro-organisms were explained upon entirely different grounds. Thus the distinction was made between organized ferments and unorganized ferments or enzyms, the former term being practically synonymous with living cells. Pasteur was the first to show that different types of fermentation were caused by specific organisms, and his epoch-making researches revolutionized the contemporary theories regarding fermentation. The former distinction between organized and unorganized ferments was finally broken down in quite recent years by Buchner. Claude Bernard had already suspected that the transformation of sugar into alcohol and carbon dioxid was due to the presence of an enzym acting within the yeast cells, but his attempts to prepare this enzym had resulted in failure. Buchner, however, showed conclusively that alcoholic fermentation could be carried on in the absence of yeast cells by an enzym prepared from the yeast.

Such enzyms which during the life of the organism act within the cell are now designated as endo- or intra-cellular enzyms. And it is by means of intracellular enzyms, prepared from the cells by suitable methods, that many of the reactions which were once thought to be absolutely dependent upon some mysterious vital activity are now carried out in the absence of living organisms.

INTRACELLULAR AND EXTRACELLULAR ENZYMS.

The distinction between intracellular and extracellular enzyms is in the main an arbitrary one. There is evidence to believe that the same enzym may, in the earlier stages of growth of the organism, function as an intracellular enzym, and later be liberated into the substratum as an extracellular enzym. In the case of yeast, the amount of active sugar-fermenting enzym or "zymase" set free in the medium during the normal course of organic growth is very small, the fermentation taking place mainly within the cells. This would account for the failure of the earlier observers to find any enzymatic activity in the medium after the cells had been removed. With molds the case is somewhat different. As the fruiting stage is reached a considerable amount of enzym is liberated into the medium, and there is no particular reason for regarding this enzym as different in origin and character from that remaining in the cell. For the sake of convenience, however, the distinction will be adhered to in this paper, as the work deals with the enzyms obtained directly from the mycelium.

The intracellular enzyms of certain groups of the lower plants have been investigated to a considerable extent, particularly the yeasts [a] and bacteria.[b] The researches on the enzyms of molds, however, have been of a more or less desultory nature, and the results, which are scattered over various chemical, biological, botanical, and mycological journals, have not as yet been collected. A great deal of this work has been carried out with organisms which have not been properly identified or where great confusion exists in the nomenclature. The results are in many cases somewhat uncertain, especially where insufficient data are given to make possible a repetition of the experiments. This point will be more fully discussed in the subsequent pages.

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[a] Meisenheimer, J. Ueber die chemischen Vorgänge bei den als Enzymreaktionen erkannten Gärungen. Biochemisches Centralblatt, 1907, Band 6, No 16/17, pp. 621-633. Leipzig, 1907.

[b] Fuhrmann, F. Vorlesungen über Bakterienenzyme. Jena, 1907.

MOLDS IN GENERAL.

A study of fungi in general would, of course, be far beyond the scope of this paper. One great group, usually called the Hyphomycetes, contains many species with wide distribution as saprophytes upon all kinds of substrata. In this group are described some 5,000 species with diverse relationship but held together by one common character—lack of sexual reproduction. Certain of these cosmopolitan groups of species together with certain of the Phycomyces make up the aggregation to which the term "molds" is commonly applied. Phycomyces or conjugating fungi include the well-known Mucors, the Peronosporeæ or rotting molds, the Cystopi or white rusts, and many other parasitic fungi, such as the Saprolegniaceæ or fish molds, and the Entomophthoraceæ or insect molds. Two other orders, the Uredineæ or rust fungi and the Ustilagineæ or smut fungi, are likewise of great economic importance. Very few of these molds have ever been studied from the standpoint of biological chemistry. The great bulk of the work has been taxonomic and pathological in character.

PENICILLIUM AND ASPERGILLUS.

The organisms selected for investigation by chemists not having an intimate knowledge of mycology would naturally be those of wide distribution and omnivorous habits. In some of the earliest work, when a mold culture was desired, the medium was simply left exposed to the air until spontaneous inoculation ensued from the spores floating about in the atmosphere. Cultures obtained in this way usually consisted of various species of two genera, *Penicillium* and *Aspergillus*, which are placed by Engler and Prantl in the family Aspergillaceæ. This family grouping is based upon certain species of each group for which sexual stages have been found, without necessarily implying that all described species of these two genera belong together. In fact, as these two genera are at present constituted, most of the described species belong rather to the Hyphomycetes than to the Aspergillaceæ as considered by Engler and Prantl.[a]

The main morphological difference between *Aspergillus* and *Penicillium* is in the appearance of the fruit bodies. In *Aspergillus* the aerial filaments bearing the spores are generally formed of a single cell which swells out at the end into a sphere. Numerous cells radiate from this sphere, each bearing one or more chains of spores. In *Penicillium*, on the other hand, the aerial filaments send out branches which ramify again, forming conidiiferous cells, and these terminate in chains of spores. The general appearance is that of a broom, whence the name *Penicillium.* While various species of these two genera may be closely related morphologically, they often show wide chemical differences. In fact some of them may be distinguished more readily by their chemical properties than by the aid of a microscope.

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[a] Engler, A., and Prantl, K. Die natürlichen Pflanzenfamilien. Leipzig, 1887.

DISTRIBUTION AND ECONOMIC IMPORTANCE.

Penicillium and *Aspergillus* are both very widely distributed in nature. Almost any nutritive substance which contains a sufficient amount of moisture and is not positively toxic will, if left exposed to the air for a short time, become infected with species of one or both of these genera. Everyone is familiar with the appearance of moldy bread, fruit, or cheese. And almost any solution containing carbon compounds which do not have antiseptic properties soon becomes covered with a growth of these molds unless special precautions are taken to sterilize the fluid and prevent access of spores. Many of these molds grow equally well, if not better, upon liquid than upon solid media, and in this respect present marked advantages over the Mucors for chemical studies.

Not only are the Penicillia and Aspergilli widely distributed in nature, but some of the species are of great economic importance. Two species of *Penicillium* are important factors in the cheese industry. The varieties of cheese known as Roquefort, Gorgonzola, and Stilton owe their peculiar flavor to a green *Penicillium* (*P. roqueforti*) which grows in the numerous crevices and air spaces occurring throughout the cheese. Camembert cheese, which is manufactured both in France and in this country, is ripened by another *Penicillium* (*P. camemberti*), which grows upon the surface and secretes enzyms into the curd mass. The rotting of oranges is due principally to *P. italicum* and *P. digitatum.* The mold found upon rotten apples is almost invariably *P. expansum.* In fact, as Thom has pointed out, many of these organisms are so intimately associated with some particular habitat that it is quite easy to obtain a pure culture simply by making a careful transfer. Several species of this genus, especially *P. brevicaule,* possess the power of transforming arsenical compounds into diethylarsin, which can be recognized even in the minutest traces by its characteristic garlic odor. By virtue of this property *P. brevicaule* has been employed very successfully as a biological test for arsenic. One-hundredth of a milligram of arsenic can be recognized in this way with certainty, the test being considered fully as delicate as Marsh's test.

The Aspergilli are of less economic importance than the Penicillia. The strong amylolytic power of some of the species is made use of in oriental countries. *Aspergillus oryzæ* is thus extensively used in the preparation of an alcoholic beverage called koji. This is made from rice by steaming the grains and inoculating with the mold, which soon converts the starch into sugar. At the same time yeast develops, and this transforms the sugar into alcohol and carbon dioxid. Another species, *A. wentii*, is used in Java for making a peculiar condiment or sauce from rice. *A. oryzæ*, after it has been cultivated on starch, then extracted with water, and precipitated with alcohol, constitutes the "Taka diastase" of commerce. Certain species of *Aspergillus* are of pathological importance on account of their parasitic habits. Thus *A. fumigatus* has frequently been found growing in the external auditory passage.

ADAPTABILITY TO ARTIFICIAL CULTURE.

Many of the species of these two genera are particularly well adapted for chemical research on account of the readiness with which they thrive upon synthetic culture media. This is undoubtedly the reason why they have been chosen by so many investigators as a basis for chemical experiments. Where a complex medium of uncertain composition, such as bean decoction, meat extract, or the like, has to be used in order to obtain a suitable growth of mold, the changes that take place in the medium can not easily be followed. This difficulty is entirely avoided by the use of a synthetic medium of known composition. In such a medium, where there is a single source of carbon or nitrogen, the carbon or nitrogen assimilation of the mold can be studied by simply varying the carbonaceous or nitrogenous constituent of the medium. As no other molds grow with such facility upon media of this character, these two genera have been used almost exclusively in such experiments.

The readiness with which these molds grow upon fluid media has already been mentioned. When it is desirable to separate the mycelium completely from the substratum, a liquid medium is practically essential. This is particularly true if determinations are to be made upon the mold itself to see what constituents of the media have entered into the actual composition of the fungus. Solid particles adhering to the mycelium would introduce a considerable error. A liquid medium, on the other hand, can be washed out completely without much trouble. The same is equally true if the mold is to be used subsequently in enzym experiments, where qualitative tests would be vitiated by the presence of a protein or carbohydrate introduced in this way. Thus for enzym experiments the Penicillia and Aspergilli are preeminently adapted.

SOURCES OF CARBON AND NITROGEN.

In the foregoing pages allusion was made to the polyphagous habits of *Penicillium* and *Aspergillus*. A number of investigators have made a careful study of the nutrition of these molds. The presence of proteins or highly complex nitrogenous substances is by no means necessary in the culture medium. The molds are able to synthesize their protoplasm from such simple substances as carbohydrates and nitrates or ammonium salts. While the sources of nitrogen and carbon may be varied within wide limits, the presence of inorganic salts in small amount is necessary. Most of the synthetic media commonly used in cultural work consist, therefore, of a dilute solution of these inorganic salts, to which has been added a carbohydrate, such as ordinary cane sugar, and a nitrogenous body varying in complexity from the nitrate of an alkali metal to a mixture of proteoses and peptones or gelatin. Nitrogen in some combined form is essential, for the molds can not utilize atmospheric nitrogen sufficiently to produce a normal colony. Often a slight growth of mold has been observed in the absence of nitrogenous compounds, but this is to be regarded as due rather to the reserve protein present in the spore itself or to a small amount of nitrogenous substance transferred to the medium during inoculation.

Many of the media in common use are unnecessarily complex. Raulin's fluid has been used perhaps more extensively than any other for cultivating molds. The inorganic constituents of this medium are quite numerous. The nitrogen is supplied in the form of ammonium nitrate and the carbon in the form of cane sugar, while free tartaric acid is added mainly to prevent the growth of bacteria. Raulin found that the yield of mold was diminished to a greater or less extent when any one of the inorganic constituents was omitted from the medium. But as later work has shown, very few of these mineral salts are really necessary. The media introduced by Wehmer and Czapek are much simpler in this respect, yet the fungus growth is, to all appearances, equally good. Some of the metallic elements are needed in such minute traces that the molds can undoubtedly make up any deficiencies of the medium from the glass of the containing vessel.

A great variety of nitrogen compounds may serve as food material for *Penicillium* and *Aspergillus*. A glance at the formulas of some of the well-known fluid culture media will show that nitrates and ammonium salts can furnish all of the nitrogen. With these molds there seems to be no particular advantage in using organic compounds of nitrogen, although the molds can utilize amids, amino acids, peptones, and some heterocycles. In the case of amino acids, according to Emmerling, the amino group must be in the alpha position with respect to the carboxyl, or else attached to alternate carbon atoms. Thus beta-amino acids are unavailable as nutritive material, while gamma-aminobutyric acid yields a good crop of mold. Czapek found that *Aspergillus niger* could not utilize hydrazins, oxims, basic heterocycles, or cyanogen compounds. The writer has likewise repeatedly failed to obtain any growth of mold upon nitrites, or the salts of hydrazin or hydroxylamin. The mold does have the power of utilizing the nitrogen of certain heterocycles, such as succinimid and nicotinic acid.

The source of carbon can likewise be varied greatly. Emmerling has shown that the molds grow luxuriantly upon a great variety of pentoses and hexoses. According to Reinke the ordinary organic acids, except carbonic, oxalic, and formic, when in combination with bases, are assimilated by *P. glaucum.* Moreover, cyclic compounds, such as parabanic and benzoic acids, can supply the mold with carbon. Diakonow goes still further and states that even formic acid can be utilized by *Penicillium.* Hasselbring tested a number of carbon compounds with respect to their availability as sources of carbon, and found that *P. glaucum* assimilates alcohol, acetic acid, and substances from which the group CH_3COO— is easily derived. According to Czapek the benzene ring is more readily attacked when it bears a number of hydroxyl groups, and such substances as quercite and quinic acid are easily assimilated. A great many of the substances, however, which are recorded in the literature as sources of carbon, yield only a scanty growth, which often fails to fructify. To secure a good thrifty colony, a substance containing one or more asymmetric carbon atoms seems to be necessary, and this is probably the reason why the sugars, from the trioses to the trisaccharids, are sources of carbon par excellence.

Since these two genera of molds, as all other fungi, contain no chlorophyll, they are obviously incapable of carrying out any photosynthesis. They must therefore derive their energy entirely from the chemical reactions which they institute in the culture medium. In other words, the medium must contain sufficient oxidizable substance, or substance capable of undergoing exothermic changes, to render the organism independent of solar energy. The chemical changes must of necessity be quite profound in order to meet these requirements. *Aspergillus niger* is said to have the power of converting one-third its weight of sugar into volatile products in a single day. While it destroys a smaller quantity of sugar than does yeast, it uses up the sugar more completely by combining it with oxygen. Assuming that the final products are carbon dioxid and water, the same amount of energy would be derived from one-twentieth the amount of sugar that the yeast consumes.

CHANGES IN THE CULTURE MEDIUM.

Some of the changes produced in the medium during the development of mold can not fail to be noticed by the most casual observer. For example, the change in color is usually quite pronounced. In some instances this change in color, or rather production of color, is so striking and characteristic as to form the basis for the specific name of the fungus. Thus *P. purpurogenum* colors the medium a brilliant reddish purple, and *P. chrysogenum* under certain conditions colors it a beautiful golden yellow. Nearly all the species when grown upon a colorless medium containing carbohydrate give rise to a brownish color, due probably to the formation of humus substances.

Other chemical changes, though not so easily detected by the eye, are none the less striking. If the reaction is at first acid to litmus, on account of the presence of free organic acid or acid phosphates, many species of *Penicillium* destroy the acid or else give rise to basic products, leaving the medium neutral or alkaline to litmus, though rarely alkaline to phenolphthalein. Other species produce acid substances and the substratum remains strongly acid to litmus. This seems to be particularly true of some of the Aspergilli. The cause of this acidity has been found by Duclaux to be due to the formation of oxalic acid. This is an intermediate product in the oxidation of carbohydrate, and if the mold is removed at the proper time a large yield of oxalic acid may be obtained. Wehmer succeeded in obtaining 80 per cent of the theoretical yield by the incomplete oxidation of cane sugar by means of *A. niger*. In the presence of a calcium salt the oxalic acid is removed from solution as fast as it is formed and can then undergo no further oxidation. Under ordinary conditions, however, the oxalic acid accumulates only so long as an excess of sugar remains, and when this becomes scarce, the oxalic acid in turn is burned up. The Penicillia, on the other hand, do not seem to have the power of producing oxalic acid to so considerable an extent; but Wehmer has shown that certain of the species produce citric acid. The citric acid is likewise an intermediate product which finally disappears, and it apparently serves the same purpose as the oxalic acid, namely, that of controlling within certain limits the reaction of the medium.

SEPARATION OF RACEMIC MIXTURES.

In connection with the effect of fungus growth upon the organic constituents of the medium, the peculiar selective power of these organisms is worthy of mention. *P. glaucum* and *A. niger* have been used by a number of investigators for preparing optically active substances from racemic mixtures. This property was first observed in 1860 by Pasteur. He prepared a solution containing the ammonium salt of racemic acid and a small amount of inorganic phosphate and inoculated it with spores of *P. glaucum*. After the mold had grown for a time the solution became levo-rotatory. The dextro acid was found to be completely destroyed, while the levo acid remained intact. Le Bel used the two molds for preparing d-methylpropylcarbinol and l-propylglycol from the respective racemic mixtures. His cultures were, however, contaminated with yeasts and bacteria. In 1882 Lewkowitsch prepared d-mandelic acid from the racemic mixture by means of *P. glaucum.* Inactive lactic acid was converted in the same way into the levo form by Linossier. By the same method Schulze succeeded in preparing optically active leucin and glutaminic acid from the inactive mixtures. The amino acid remaining is always the one that does not occur in nature, and Schulze claims that these amino acids can be obtained in the form isomeric with the original substance by racemizing and then subjecting to the action of *P. glaucum.* Buchner notes that *P. glaucum* and *A. niger* grow readily upon the ammonium salts of fumaric, but not on those of maleic acid, and gives us a method of separating isomeric mixtures where the isomerism is due to the presence of a double bond. In 1899 Fischer inoculated a solution containing d-alanin and inorganic salts with *A. niger* and found that 10 per cent of the d-alanin was used up in fifteen days, the remaining solution being levo-gyrate. And still more recently Neuberg and Mayer obtained l-cystin from the inactive mixture by *A. niger.*

Other chemical changes in the medium, especially those of the nature of hydrolytic cleavage, such as the breaking down of proteins, the inversion of disaccharids, and the liberation of ammonia from amids, are due to enzyms; which can be made to act independently of the cells themselves. These changes will be discussed later on under the subject of enzyms.

BEHAVIOR TOWARD ANTISEPTICS.

Before entering upon a discussion of enzyms, however, it might be well to mention very briefly the behavior of these molds toward antiseptics. This problem has been studied by a great number of investigators, and so many observations have been reported that it would be out of the question to give a detailed discussion here. It is well known that molds are far less sensitive to antiseptics than are bacteria. The literature contains many observations of instances where these molds were found growing in solutions of substances known to possess germicidal properties. A table showing the relative toxicity of a number of the well-known antiseptics has been worked out by Malenkowitsch. According to his observations, the salts of the heavy metals are most toxic, while some of the organic antiseptics must be present in considerable amount before growth of mold is inhibited. It appears that those substances are most toxic which act oligodynamically, or those which, like chloroform, toluene, and ether, have a solvent action upon fats and lipoids.

REVIEW OF PREVIOUSLY PUBLISHED WORK ON THE ENZYMS OF PENICILLIUM AND ASPERGILLUS.

Although the effect of *Penicillium* and *Aspergillus* upon various natural substrata and upon synthetic culture media had been observed for many years, the changes were not ascribed to the action of enzyms. This may be explained upon the ground that the enzyms are for the most part intracellular. Attempts to demonstrate the presence of an enzym in the substratum after removing the mold would therefore result in failure. The secretion of enzyms into the culture medium does not ordinarily occur to any appreciable extent during the active vegetative period of the mold, but at the time of fructification, and subsequently, some of the enzyms are liberated. Pathological conditions—such as exhaustion of the medium, lack of aeration, or prevention of fructification—have a similar effect. The liberation of the enzym has been regarded by a number of investigators as due rather to the disintegration of some of the cells than to secretion by active living cells. Any procedure that tends to produce plasmolysis of the cells is therefore accompanied by liberation of the enzyms. Various methods of preparing enzym solutions from molds are based upon this principle.

THE VARIOUS METHODS OF PREPARING THE ENZYMS.

A method devised by Duclaux and used in a number of his experiments consists in allowing the mold to grow upon Raulin's fluid until spores begin to form, then siphoning off the medium and replacing it several times by distilled water, and then, after the last traces of nutrient material have been washed out, allowing the mycelium to remain in contact with the distilled water for several hours. In this way the cells are made to yield up their enzyms to the water.

Another method, used by Bourquelot, is based upon the destruction of the cell wall by more heroic treatment. It consists in removing the mold at the appropriate time from the culture medium and triturating it in a mortar with sand and chloroform water. The extract thus obtained is filtered and may be used either directly or after precipitation with alcohol.

A third method consists in drying the mold at a low temperature (40° C.) and grinding the resulting product to fine powder. The advantage of this method is that the enzyms in the dry state retain their activity much longer. A solution of the enzyms may be obtained at any time from this powder by simply extracting with water and filtering. These three methods have been employed in all of the earlier work on mold enzyms.

Two other methods introduced by Buchner have been used extensively in preparing yeast enzyms, but have only very recently been applied to molds. One of these makes use of the hydraulic press, which squeezes out the liquid contents of the cells. The other is the simple dehydration of the organism by means of acetone and ether. The latter method will be described more fully in the experimental part of this paper.

Most of the investigators have examined the enzyms at the time of fructification of the mold. This varies considerably with the individual species. Some of the green Penicillia produce spores within three days, while with other Penicillia the conidia may not appear until after a week or ten days. There appears to be good reason for preparing the enzyms at this particular stage if the maximum activity is desired. It can be shown experimentally that the amount of intracellular enzym rapidly diminishes after fructification, and at the same time the enzyms begin to appear in the substratum. The proper time to prepare the intracellular enzyms is, therefore, just before fructification. The explanation given by some authors is that fructification is accompanied by a disintegration of mycelial cells, and the enzyms contained in them are thus set free. Histologists claim, however, that there is no evidence of any disintegration of cells, at least not until after the crop of spores is fully developed. It is not unreasonable to suppose that during the vegetative growth of the organism the enzyms are constantly being produced and stored up within the cells. Then when spore formation begins it is accompanied by increased metabolic activity, and in response to the plant's greater need of easily assimilable nutrients the enzyms are turned loose to act in the medium. Hence to obtain the greatest yield of endoenzyms, they must be caught before they escape as ectoenzyms.

THE PARTICULAR ENZYMS FOUND.

Having briefly mentioned the general methods by which the intracellular enzyms of *Penicillium* and *Aspergillus* may be obtained, we now turn our attention to a consideration of the individual enzyms that have been found in these molds. With our present knowledge of zymology it is impossible to separate individual enzyms from a mixture. The only instance on record where a satisfactory separation has been accomplished is that described by Vines, where he isolated pepsin and erepsin from the mixture occurring naturally in plants. Most of the enzyms, however, are so closely similar in their solubility and behavior toward reagents that attempts to separate them have resulted in failure. But by preparing enzyms from different sources we can obtain preparations that are active toward certain zymolytes and inactive toward others, and in this way our knowledge of the specificity of enzyms has been acquired. When we have to deal with a mixture, therefore, as in the case of mold extracts, the different types of enzym reactions can be studied separately by testing the activity of the mixture toward different substrata, and from these observations inferences can then be drawn as to the presence of the individual enzym responsible for these reactions.

The enzymic activities ascribed to *Penicillium* and *Aspergillus* species are briefly stated in Wehmer's review. As this author does not, however, give any description of the methods employed by other investigators in their search for enzyms, it was thought advisable to discuss the literature somewhat in detail in this place. The methods and results will be given as described by the authors and with very little comment. Later on it will be shown that the results obtained by the different investigators are not strictly comparable with each other nor with those obtained by the writer, owing to the fact that different species were used. The differences are, however, quantitative rather than qualitative.

PROTEASE.

An examination of the literature will show at once that the proteolytic enzym of *Aspergillus* and *Penicillium* has received more attention from previous investigators than any other single enzym. Yet there are so many conflicting statements that the identity of this enzym is by no means established. Hansen was the first to turn his attention to this subject. He found that when *P. glaucum* was grown upon 6 per cent gelatin the latter was rapidly liquefied. He also made a glycerol extract of the mold just after the liquefaction of the medium had begun and only a few spores had formed. This extract was found to liquefy gelatin in neutral solution more rapidly than in the presence of acid. Attempts to prepare the enzym in the dry state failed. Several years later Wehmer confirmed the observations of Hansen. He found that various species of *Penicillium* and *Aspergillus* liquefy the gelatin of culture media to a considerable distance beyond the colony itself or beyond the reach of the mycelium. The liquefaction was greatest when gelatin was the only source of carbon.

Bourquelot studied the proteolytic enzym of *A. niger* after it had been cultivated on Raulin's fluid. An extract of the mycelium was prepared by triturating with sand and chloroform water. To one portion of this extract a few flocks of fibrin were added, while in another portion were placed a few pieces of coagulated egg white, and both were warmed to 40° C. In both cases there was an unmistakable formation of peptone. The filtered liquid gave only a slight cloudiness with nitric acid, and a characteristic red biuret reaction. Bourquelot concludes, therefore, that the extract contained trypsin. In testing for pepsin he followed the same procedure except that the liquid was made 0.2 per cent acid with hydrochloric acid. There was a disintegration of the fibrin and a slight solvent action upon the albumin. But the tests for peptone were negative, and the author considers that the presence of pepsin is very doubtful. Bourquelot noticed also that cultures of *A. niger* possess to a limited extent the property of liquefying gelatin.

In 1900 Malfitano made a more detailed study of the protease of *A. niger.* He used Fermi's liquid gelatin method. The method as adapted by Malfitano consists in mixing 10 c. c. of the solution to be tested with 5 c. c. of a warm solution of 20 per cent gelatin containing 0.2 per cent thymol. At room temperature the mixture is solid, but at 35° C. it is liquid. The mixture is left in the thermostat for a given length of time, then cooled down to 15° C. If any digestion has occurred, the solution remains liquid, otherwise it quickly solidifies. By means of this method Malfitano was able to make a number of interesting observations. He found that the enzym was active only in acid solution. He studied the enzym in the medium (Raulin's fluid) as well as in the mycelium. In the medium the enzym showed its greatest activity when the mycelium had reached its maximum growth. The quantity of enzym was found to be dependent upon the intensity of development of mycelium and upon the degree of maturity. Conditions of aeration or temperature had no direct influence, except in so far as they influenced the growth of mold. He found that when cubes of coagulated egg white or flocks of fibrin were introduced into Raulin's fluid in place of the nitrogen-containing salts they failed to yield any growth of mold. Casein and gelatin furnished an available source of nitrogen, and were still more readily attacked in the presence of other nitrogenous food material.

Malfitano studied also the proteolytic enzym contained in the mycelium. The enzym solution was prepared by Bourquelot's method of grinding the mycelium with sand and chloroform water. The activity of the enzym in the mycelium was found to reach its maximum at the time of spore production, then the cells began to yield up their enzym to the medium, and the enzym in the medium increased at the expense of that in the mycelium.

In a second paper Malfitano describes an experiment in which *A. niger* was allowed to develop until the time of sporulation, then the mycelium was removed from the medium and dried at 35° C. An extract of this product digested gelatin readily, and the formation of protogelatose, deuterogelatose, and gelatin peptone was demonstrated. The extract would not digest Mett's tubes of coagulated egg albumin nor boiled fibrin. Fresh fibrin, on the other hand, was digested in the presence of one-hundredth normal acid, while the fibrin in the control test merely swelled. Milk first curdled and then digested, but the coagulum first formed did not disappear completely. The presence of caseose and peptone was demonstrated in the digested milk.

In the same year Steffens reported some observations on the relation between the formation of proteolytic enzym and the nutrition of mold. He used four species of *Penicillium* (*P. glaucum*, *P. luteum*, *P. rubrum*, and *P. italicum*) and five of *Aspergillus* (*A. niger*, *A. glaucus*, *A. oryzæ*, *A. wentii*, and *A. fumigatus*). These were all grown upon five different media, as follows: Raulin's fluid, starch paste, starch paste and peptone, diluted milk, and egg white and water. After eight days the mold was ground with sand and chloroform water, filtered, and the enzym precipitated by alcohol. Its digestive action was tried upon egg albumin, casein, fibrin, and "vegetable casein" in the presence of 0.2 per cent hydrochloric acid and 0.2 per cent sodium carbonate. The author used the biuret test to determine whether digestion had taken place. This he considers more delicate than Fermi's gelatin method, for it gave positive results where Fermi's test was negative. Needless to say, he obtained evidence of digestion both in acid and alkaline solution of all the four proteins used. No mention is made of control tests with boiled enzym or with the enzym solution alone.

A study of the effects of living molds upon proteins was made by Butkewitsch. He found that when *A. niger* was grown upon "Witte peptone" most of the nitrogen was converted into ammonia, which neutralized the oxalic acid produced simultaneously. Other nitrogenous substances, among which were leucin and tyrosin, were also formed, but in relatively small amount. *P. glaucum*, on the other hand, produced much less ammonia and relatively more amino acids. As no oxalic acid was produced by this organism, the reaction of the medium became alkaline. Butkewitsch claims that the proteolysis is due to the secretion of an enzym resembling trypsin. This enzym is contained not only in the mycelium, but also in the culture medium. When the mold is grown upon peptone, more proteolytic enzym is formed than when the source of nitrogen is ammonium tartrate. The further decomposition of the amino acids into ammonia is an independent process which serves the purpose of maintaining the proper reaction of the medium. It may be influenced in a variety of ways. If calcium carbonate is added to the *Aspergillus* culture, the oxalic acid combines with it and prevents its accumulation in the medium. Consequently there is no need of ammonia, and less is produced. These conditions may be reversed in the case of *Penicillium*. If phosphoric acid is added to the *Penicillium* culture, which normally produces very little ammonia, there is a marked increase in ammonia formed.

A comparison of the results obtained by these authors would indicate that the organisms studied contain a protease that acts readily upon gelatin and less readily upon fibrin and egg albumin. In spite of conflicting statements regarding the effect upon fibrin and egg albumin, the authors have held to the view that the enzym is of the nature of trypsin.

NUCLEASE.

The effect of cultures and enzym preparations of *A. niger* and *P. glaucum* on thymonucleic acid was studied by Iwanoff. He found that while the sodium salt of thymonucleic acid could not supply the molds with carbon, it furnished a good source of nitrogen and phosphorus. The molds were accordingly grown upon a nucleic acid solution to which had been added cane sugar, magnesium sulphate, and potassium chlorid. After several days the culture fluid was found to give a precipitate of purins with ammoniacal silver nitrate, and a copious precipitate with magnesium mixture. In one experiment which was conducted for thirty-two days the decomposition of the nucleic acid was complete, 70 per cent of the phosphorus appearing as inorganic phosphate and the other 30 per cent entering into the cell structure of the fungus. The author showed by separate experiment that the cleavage could not have been caused by the oxalic acid produced by the mold. To prove conclusively that it was due to an enzym contained in the mycelium, the latter was triturated to a paste with infusorial earth and added to a solution of nucleic acid containing 150 mg. P_2O_5 and chloroform as an antiseptic. In two weeks 7 mg. P_2O_5 and 12.7 mg. nitrogen as purin bases were liberated. A control experiment with boiled enzym gave no purin precipitate. The author claims that this enzym, which he calls nuclease, is entirely distinct from the proteolytic enzym, for the mycelium paste did not liquefy gelatin.

AMIDASE.

An extensive study of the amidase of *A. niger* was made by Shibata in 1904. Previous to that time the power of fungi to liberate ammonia from amids and amins had been observed by Butkewitsch and others, but only in culture media. Shibata now succeeded in hydrolyzing these compounds by means of enzym preparations. *A. niger* was grown upon a medium containing peptone, sugar, and inorganic salts. Two methods were used in preparing the enzym. The mycelium was collected and forced through a sieve, then it was either allowed to dry at ordinary temperature or else treated with acetone and ether according to Albert and Buchner's method for yeast. A portion of the powder thus obtained was mixed with a solution of the substance to be tested, toluene added, and the mixture left in the thermostat for several days. The ammonia was then determined by distillation with magnesium oxid, or in the case of the less stable urea derivatives and acid amids the method of Schlössing was employed. Urea and acetamid yielded the most ammonia, though appreciable amounts were also obtained with biuret, oxamid, benzamid, asparagin, and alanin. Urethan, guanidin, allantoin, and uric acid gave no ammonia at all. Hippuric acid was decomposed into benzoic acid and glycocoll.

In repeating these experiments with an acetone preparation of yeast Pringsheim obtained negative results. A suspension of yeast, however, readily liberated ammonia from amino compounds. It is evident therefore that the amidase of *Aspergillus niger* is more resistant to acetone and ether than that of yeast.

LIPASE.

The fact that molds can utilize fats as well as carbohydrates was first noticed by Van Tieghem. He found that when a porous body that had previously been soaked in water was immersed in an animal or vegetable oil the surface of this body soon became covered with a growth of mold, the predominating species of which was *P. glaucum*, and, strange to say, the mold thrived and produced normal fructification, although apparently excluded from air by the layer of oil. At first there seemed to be no change in the oil, but after a time a white crystalline mat began to form where the oil was in contact with the mycelium. The crystals were needle-shaped, and were at first very small, but they increased in size until they attained a length of 1 to 2 mm. The author states that these crystals consisted of fatty acids, produced by hydrolysis of the fat.

Gérard cultivated *P. glaucum* upon Raulin's fluid to which a little monobutyrin had been added, and noticed that there was a liberation of butyric acid. The fat-splitting power of this organism was shown by him to be due to an enzym contained in the mycelium. An aqueous extract of the mold had the power of hydrolyzing monobutyrin. To 100 c. c. of a 2 per cent monobutyrin solution he added 30 c. c. of the mold extract. The number of cubic centimeters of decinormal alkali that it required to neutralize this solution increased from 0.4 in the control to 2.5 in the digestion experiment in six days. This represents less than 3 per cent of the theoretical yield of butyric acid, and the lipolytic action must therefore have been quite feeble.

Camus confirmed the observation of Gérard, and found also some slight evidence of lipolytic action in *A. niger*. Cultures of *P. glaucum* upon monobutyrin grew poorly, and the lipolytic action was not very marked. The medium did, however, increase in acidity, and this the author attributes to the liberation of butyric acid. In the case of *A. niger* the lipolytic action was still more feeble.

Garnier grew *A. niger* upon a modified Raulin's fluid at 35° C., then filtered off the mycelium and allowed it to act upon a solution of monobutyrin in the presence of chloroform. A short time after inoculation, just as the spores began to appear, there was evidence of a small amount of lipolytic enzym. The amount of this enzym quickly diminished and remained very small for about eleven days. At the end of that time the activity began to increase, without, however, becoming very marked. It reached its maximum on the fifteenth day, after which it remained practically constant for about two months. The same author studied the lipase of *A. fumigatus* and found it to be very feeble. Two days after inoculation a slight lipolytic action was observed, but this disappeared at the time of fructification. At the end of twelve days, when the surface of the colony was completely covered with spores, the lipase reappeared, but only in small amount. *A. flavus* and *A. glaucus* behaved in the same way, though the latter yielded somewhat more lipase.

The action of *P. glaucum* on butter fat was studied by Laxa. He noticed that butter inoculated with this organism developed considerable acidity. By extracting the mycelium obtained by inoculating this organism on media containing asparagin and lactic acid he obtained an enzym which hydrolyzed monobutyrin.

In more recent years Deleano has studied the lipase of several molds. He cultivated *P. glaucum*, *A. niger*, and *A. flavus* on Raulin's fluid for seventeen days. The mycelium was then filtered off and dried at 37° C. The medium upon which the molds had grown showed no lipolytic power. Various solutions were used in extracting the dried mold. Physiological saline with 5 per cent glycerol, 1 per cent sodium hydroxid, and 50 per cent glycerol extracted no lipase. When the powder was extracted for two days in the thermostat with 1 per cent sodium phosphate and 0.5 per cent alcohol some lipase was obtained. The extract made from *A. niger* in this way acted strongly upon tributyrin, feebly upon monobutyrin, while it had no action at all upon ethyl butyrate. *A. flavus* showed a feeble action upon tributyrin and ethyl butyrate and none upon monobutyrin. *P. glaucum* failed to hydrolyze any of the three esters. From these experiments the author suggests the possibility of specific enzyms for different esters.

EMULSIN.

A glucosid-splitting enzym was observed almost simultaneously in 1893 by Gérard and by Bourquelot in two different molds. Gérard prepared an extract from the mycelium of *P. glaucum* by trituration with sand, and precipitated the enzyms from this extract by the addition of alcohol. After dissolving and reprecipitating, the product was washed with ether and dried in vacuo. One-hundredth of a gram of this preparation hydrolyzed 10 c. c. of a 1 per cent amygdalin solution completely into glucose, benzaldehyde, and hydrocyanic acid in twenty-four hours. Salicin was likewise completely hydrolyzed in the same length of time.

Bourquelot prepared his enzym from *A. niger* by growing the organism on Raulin's medium until the time of fructification, then floating the mycelium on distilled water for several days. The solution obtained in this way when allowed to act upon amygdalin gave the characteristic odor of benzaldehyde within an hour. Bourquelot and Hérissey found that not only could amygdalin be hydrolyzed, but also salicin, coniferin, arbutin, esculin, populin, and phlorhizin. On the other hand, some of the less soluble glucosids, such as solanin, hesperidin, convallamarin, convolvulin, digitalin, and jalapin remained unaltered.

A comparison of the emulsin of *A. niger* with that of almonds was made by Hérissey. He tested the action of the enzym prepared from both sources on a number of glucosids. While the enzym from *A. niger* was found to decompose populin and phlorhizin, the emulsin of almonds had no effect upon these two substances. There was also a decided difference in the rate at which the two enzyms decomposed other glucosids. The enzym of *Aspergillus* acted more energetically upon arbutin than upon any other glucosid, while the reverse was found to be the case with the emulsin of almonds. Further investigation of the glucosid-splitting power of *A. niger* showed that the formation of this enzym could be entirely suppressed by an excess of ammonium nitrate in the medium, but upon replacing this solution by water an abundance of emulsin was formed. The author believes that a condition of starvation may result in the formation of enzyms that were previously absent.

The glucosid-splitting power of the same organism as well as that of *A. glaucus* and *P. glaucum* was investigated by Puriewitsch. This author experimented with the living mycelium. The mold was grown upon Raulin's fluid, then the liquid removed and replaced two or three times by distilled water and finally by a solution of the glucosid. When salicin was used, a cleavage was evident in fifteen to twenty hours. The salicin decomposed with the formation of saligenin which gives a blue color with ferric chlorid. The intensity of the color increased with the time during which the mold was allowed to act. No glucose, however, could be demonstrated by Fehling's solution, and it was evidently used up by the mold as fast as it was formed. Similar results were obtained with helicin, arbutin, coniferin, esculin, phlorhizin, and hesperidin, though of these only the first two give products that can easily be detected by odor or color reactions. Amygdalin behaved quite differently according as the living mycelium or an extract was used. Upon solutions of amygdalin the mold grew well, but no odor of benzaldehyde and no hydrocyanic acid could be detected. The nitrogen was liberated in the form of ammonium salts. When, however, an extract of the mycelium was used, the amygdalin was rapidly decomposed into benzaldehyde, hydrocyanic acid, and glucose. The selective action of the mold is quite apparent from these experiments. The organism uses up the glucose and leaves the benzene derivative intact until the former is exhausted; then the latter is utilized also. Moreover, in the presence of a large amount of glucose or cane sugar the glucosid itself is not attacked.

The stereochemical configuration of the glucosid molecule is of importance in determining the effect of the enzym. Pottevin found that a solution of *A. niger* which had been cultivated on Raulin's fluid acted on amygdalin and on beta-d-glucosids, but not on the two methyl-galactosids nor on lactose. When, however, lactose or methyl-d-galactosid was added to the culture medium, an enzym capable of hydrolyzing them was developed.

Brunstein again took up the study of the effect of certain fungi on media containing glucosids. Among the organisms cultivated were *A. niger*, *A. oryzæ*, *A. wentii*, *A. glaucus*, and *P. glaucum*. These were grown upon media consisting of Raulin's fluid and peptone, and after the mold had reached maturity the culture fluid was replaced by a solution of glucosid. The relative nutritive value of the different glucosids was determined by noting the increase in weight of the mycelium. Coniferin was most readily utilized, while arbutin, helicin, and salicin had a toxic effect. The latter was due to the liberation of the toxic substances hydroquinone, salicylaldehyde, and salicylalcohol from these glucosids. Certain of the organisms overcame the toxic action of salicin by oxidizing the salicylalcohol liberated to aldehyde and then to salicylic acid.

"TANNASE."

The power possessed by *Aspergillus* and *Penicillium* to hydrolyze tannin was observed as early as 1867 by Van Tieghem. This author noticed that a solution of tannin does not undergo change if it is kept saturated with carbon dioxid or is rendered sterile by heating. But when the solution is left exposed to the air, molds make their appearance and decompose the tannin into gallic acid which crystallizes out. Inoculation of tannin solutions with pure cultures of *P. glaucum* and *A. niger* gave the same phenomenon. As his attempts to decompose tannin by an extract of the mycelium were unsuccessful, he concluded that the decomposition of tannin with the formation of gallic acid was a true fermentation in Pasteur's sense, and he called it "gallic fermentation."

In 1900 Fernbach again took up the subject of gallic fermentation. He grew *A. niger* on Raulin's fluid in which the sugar had been replaced by tannin, and found that the tannin was slowly decomposed with the formation of gallic acid. An enzym preparation obtained from this culture was more active and hydrolyzed a 10 per cent solution of tannin at 50° C., so that on cooling crystals of gallic acid separated out. The mold extract filtered through a Chamberland filter and added to a sterile solution of tannin gave the same results.

Almost simultaneously with Fernbach's work, Pottevin obtained an enzym in the same way which decomposed commercial tannin into gallic acid and 12 to 15 per cent glucose. No tannase was formed unless tannin had been added to the medium on which the organism was grown.

The "tannin" used in these experiments must have been a glucosid of some sort.

ENZYMS ACTING ON CARBOHYDRATES.

It is generally understood that the complex polysaccharids, as well as the more simple disaccharids and trisaccharids, are not directly assimilable. They must first undergo a process of cleavage by which they are transformed into monosaccharids, and this is accomplished by various enzyms, each of which is specific for a particular carbohydrate. The specificity of these carbohydrate-splitting enzyms is very marked, and it is possible to prepare enzyms which act vigorously upon a single carbohydrate and no other. No one, however, has succeeded in separating mixtures of these enzyms, and where a given preparation acts upon a number of complex sugars it must be assumed that the various activities are due to individual specific enzyms. The significance of this will at once be seen in a study of the lower fungi. As the molds can utilize a great variety of complex sugars, the enzyms which decompose these sugars must all be present together, unless we are to assume that the organism possesses a certain power of adaptation by which an enzym is formed only when it is needed for the utilization of that particular substratum. With this view in mind we now turn our attention to a review of the various sugar-splitting enzyms found in *Penicillium* and *Aspergillus*.

AMYLASE.

The formation of amylase by *Aspergillus* and *Penicillium* seems to be subject to variations depending upon the species and upon the nutrient medium on which the organism is cultivated. According to Duclaux, *A. glaucus* when cultivated on solutions containing calcium lactate as the only source of carbon produces amylase but no protease nor sucrase. On the other hand, when cane sugar served as the source of carbon, sucrase was formed but no amylase. *P. glaucum* produced sucrase on both media, but the amylase was much weaker. With glycerol as the source of carbon a feeble amylase was obtained. Duclaux states also that *A. niger* does not germinate on starch, but is able to utilize starch if the mold is first cultivated on a sugar solution and the mycelium transferred to an amylaceous medium. *A. oryzæ* germinates on sugar solutions, but thrives less well upon such media than upon starch. According to Petit, *P. glaucum* and *A. niger* grow upon solutions of dextrin. The medium acquires a reducing power and maltose and dextrose may be demonstrated.

In general it may be said that the Penicillia secrete less amylase and are consequently less well adapted to grow upon starch-containing media than the Aspergilli. Hansen states that his *Penicillium* which had been cultivated on gelatin had no action at all on starch paste.

With an extract of the mycelium of *A. niger*, Bourquelot tested the amylolytic action on a 0.5 per cent paste of potato starch. After thirty hours the mixture gave no color with iodin, and with Fehling's solution a reduction was obtained corresponding to 92 per cent of the theoretical amount of glucose.

The organism which produces amylase in greatest amount is *A. oryzæ*. This is the source of the commercial "Taka diastase." A description of the preparation and properties of the enzym is given in Takamine's paper. According to this author the organism is grown on wheat bran, the mycelium extracted with water, and the extract precipitated by alcohol and dried. The resulting product is more active than malt diastase, and has the power of hydrolyzing 100 parts of starch in ten minutes.

An interesting study of the effect of the culture medium upon the formation of amylase was made by Katz. He found that when the medium contained 0.25 per cent starch *P. glaucum* hydrolyzed the starch, so that in two days the iodin test was negative. The growth on starch was not, however, luxuriant. If the medium contained 1.5 per cent cane sugar in addition to the starch the growth was more vigorous, but the disappearance of the starch was considerably delayed. Fifteen per cent sucrose or 2 per cent dextrose completely suppressed the production of amylase. This was not due to any effect upon the enzym itself, for the amylase acts readily on starch when sugar is present in this concentration. An extract of the mycelium from a culture that had been obtained with 0.25 per cent starch and 2 per cent sucrose had no effect at all upon starch. Maltose and lactose did not suppress the amylase, even if present to the extent of 10 per cent. Erythrodextrin had no effect, nor had quinic acid, glycerol, tartaric acid, or meat extract when added to the medium. The addition of peptone caused the inhibitory effect of the sugar to vanish.

The formation of amylase by *A. niger* was less susceptible to these inhibitory influences than *P. glaucum*. On solutions containing only starch, the growth was slow and the amylase rather feeble. The disappearance of the starch was accelerated by the addition of 1.5 per cent sucrose. Further addition of sucrose delayed the disappearance of the starch, but a concentration of 30 per cent did not suppress it. The mycelium yielded more amylase when the organism was grown with starch than it did in the absence of starch. From these experiments the author concludes that molds have the power of regulating the production of amylase according to the nutritive requirements, and that this power of regulation is more highly developed in *P. glaucum* than in *A. niger*.

INULASE.

Before the enzym inulase was discovered, Bourquelot had noticed that *A. niger* grew on a solution containing inulin as the only source of carbon as well as it did on cane sugar. Several years after the publication of the paper by Green,[a] Bourquelot again took up the subject of the cleavage of inulin by *A. niger*. An enzym preparation of this organism obtained by Duclaux's method was found to act readily upon solutions of inulin. A 1.32 per cent solution of inulin was converted in eighty-four hours at 17° C. into 1.40 per cent reducing sugar, while the rotation increased from -1.06 to -2.53. This represents complete hydrolysis. He states that yeast does not ordinarily ferment inulin solutions, but in the presence of a little inulase from *A. niger* the fermentation goes on very rapidly.

A more extended research into the inulase of *A. niger* and *P. glaucum* was made in 1903 by Dean. This author obtained the enzym by cultivating these organisms for a week on media containing inulin, filtering off the mycelium and immersing it for five minutes in a mixture of absolute alcohol and ether. The mycelium was obtained, on drying, in the form of a grayish powder. This powder readily hydrolyzed solutions of inulin, but no inulase could be detected in the culture fluid upon which the organisms had grown. The enzym was very sensitive to acids and alkalies. It was destroyed by sulphuric acid or potassium hydroxid in concentrations of 0.01 normal. The optimum temperature was determined as 55° C. The *Aspergillus* powder possessed a rather stronger inulin-splitting power than the *Penicillium* powder.

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[a] Green, J. R. On the germination of the tuber of the Jerusalem artichoke (*Helianthus tuberosus*). Annals of Botany, vol. 1, p. 233. Oxford, 1887–1888.

Saiki used a preparation of inulase obtained in this way from *A. niger*. By injecting it into the blood circulation of a rabbit he was able to prepare a serum containing antiinulase and antisucrase.

RAFFINASE.

Scheibler's discovery of a raffinose-splitting enzym in yeast led Bourquelot in 1896 to search for this enzym in *A. niger*. He mixed 50 c. c. of a 4 per cent raffinose solution with an equal volume of the culture fluid upon which this organism had grown. In four days the rotation of the mixture had decreased from 4.8 to 4.0, and a reduction of Fehling's solution obtained corresponding to 0.66 gram sugar. This represents one-third the amount yielded when raffinose is completely hydrolyzed. Bourquelot is cautious, however, about making any inferences as to whether the hydrolysis had gone beyond the formation of levulose and melibiose and decomposed the latter into dextrose and galactose.

Three years later Gillot studied raffinose with reference to its availability as a carbohydrate food for *A. niger*. He prepared a culture medium which consisted of Raulin's fluid with raffinose in place of the sucrose. As the free tartaric acid in this medium was found to hydrolyze some of the raffinose at the temperature of the autoclave, the sterilization was effected by filtering through a Chamberland filter. When this medium was inoculated with *A. niger* a colony developed and produced normal fructification. Examination of the medium every twenty-four hours showed that the reducing sugar formed rapidly increased in amount, while the amount of unchanged raffinose diminished. Oxalic acid was produced just as when cane sugar furnished the carbon. The complete disappearance of the carbohydrate the author interprets as evidence of the presence of a second enzym, "melibiase," which breaks up the melibiose first formed into dextrose and galactose.

The hydrolysis and utilization of raffinose by *P. glaucum* was next investigated by the same author. In the first series of experiments the culture medium was prepared as before. *P. glaucum* inverted the raffinose completely in four days, though some of the reducing sugar remained until the twelfth day. To prove that the hydrolysis was really due to an enzym, the mycelium was extracted by the method of Duclaux and the extract allowed to act upon raffinose. In twenty-six days the rotation had decreased from 8.8 to 6.2 and 0.324 gram glucose was formed, corresponding to 22 per cent hydrolysis. Instead of using an antiseptic in these experiments the author heated the mixture every day for two hours at 55° C. He admits the inadequacy of this method as a means of sterilization, but states that in this case it was apparently effective.

SUCRASE.

As early as 1858 Béchamp found that solutions of cane sugar containing small amounts of inorganic salts became levo-rotatory on standing, while at the same time molds developed. A solution of pure sucrose in distilled water did not undergo change, and the solution containing salts likewise remained unchanged when a little mercuric chlorid or creosote was added. The molds were not identified but were described merely as green, yellow, or white, and the mycelium as floating or submerged. There can be little doubt, however, that some of the molds at least were Penicillia or Aspergilli.

Twenty years later Gayon cultivated *P. glaucum* and *A. niger* on solutions containing cane sugar and noticed that the sugar rapidly became inverted. He noticed also an important distinction between these molds and some of the Mucors, the latter not having the power to invert cane sugar. Several years later Duclaux in studying the food value of different substances for *A. niger* likewise noted that sucrose in the medium became inverted.

The presence of an intracellular enzym responsible for the inversion of the sugar was definitely established by the experiments of Bourquelot. In his paper on maltose this author states that an aqueous extract of the mycelium of *A. niger* hydrolyzes cane sugar as well as maltose, and that the extract loses its activity on filtering through porous clay. *Penicillium* likewise yielded a mixture of enzyms, but in this case the action upon sucrose was much more energetic than that upon maltose or starch paste. In a later paper the same author showed that this enzym solution added to an equal volume of a 2.5 per cent sucrose solution gave the theoretical yield of invert sugar in twenty-four hours.

Fernbach made a detailed study of the sucrase of *A. niger*. He prepared a series of flasks, each containing the same amount of Raulin's medium, inoculated them as nearly as possible with the same number of spores, and allowed the organism to grow at 35° C. At different stages of growth the mycelium was filtered off and the filtrate made up to a definite volume, then the invert sugar was determined. In three days the inversion was nearly complete and a large proportion of the sugar had disappeared, while in four days not a trace of sugar was left. Although in two days the mold had reached half its maximum weight, no inverting enzym was found in the medium. But as soon as the medium became deficient in sugar the liberation of enzym began, and in eight days there was six times as much enzym in the medium as there was in three days. The diffusion of sucrose from the cells may be hastened by the exclusion of oxygen and consequent prevention of fructification. The author holds, therefore, that there is no genetic relationship between the formation of spores and the appearance of sucrose in the medium. This view leads to the assumption that the inversion of sugar is normally an intracellular process. Although the medium itself shows no marked sucroclastic action until fructification has begun or the liberation of enzym has been induced by pathological conditions, the mycelium contains a very active sucrase from the start. Its activity gradually increases, and as the sugar becomes exhausted the greater part of the intracellular enzym is set free and appears in the medium.

According to Fernbach, the sucrase of *A. niger* is most active in an acid medium. He constructed a table showing the amounts of different acids that must be present to produce equal effects. This table was interpreted by Kanitz, who showed that the effect of the acid was determined entirely by the concentration of hydrogen ions. The optimum concentration of hydrogen ions for the sucrase of *A. niger* is from $\frac{1}{3000}$ to $\frac{1}{300}$ normal.

MALTASE.

In his classic paper on the physiological properties of maltose Bourquelot describes a series of experiments which demonstrate the presence of a maltose-splitting enzym in *A. niger* and *P. glaucum.* He cultivated the organisms on Raulin's fluid, and when they had reached maturity he siphoned off the culture fluid and replaced it first by water and then by a solution of maltose. By noting the decrease in rotation and determining the reducing power with Fehling's solution he was able to prove that most of the maltose became converted into dextrose. Not only the living mycelium, but also an enzym obtained by precipitating an extract of the mycelium by alcohol had the power of transforming maltose into dextrose. The solution of maltase was found to lose its activity considerably when passed through a porous clay filter. *A. niger* not only continued to grow when the medium was replaced by a solution of maltose and inorganic salts, but the organism germinated readily when such a solution was inoculated with spores. The maltase of *P. glaucum* was not as active as the sucrase present in the same extract.

Bourquelot's experiments were performed without the use of an antiseptic. Ten years later Hérissey prepared a maltase from *A. niger* by floating the mycelium on distilled water. The action of the enzym was tested by the polariscope, and it was found that the addition of chloroform had no inhibitory effect.

LACTASE.

Bourquelot and Hérissey failed to find any evidence of lactase in *A. niger.* Neither the medium nor the mycelium from cultures of this organism on ordinary Raulin's fluid had any effect on milk sugar. Only one instance is on record of the occurrence of lactase in these lower fungi. Pottevin confirmed Bourquelot's observation that *A. niger* when cultivated on Raulin's fluid produced no lactase. But when the culture fluid was replaced by lactose and inorganic salts after the organism had begun to grow, the mold continued to develop and a lactose-splitting enzym was found in the mycelium. The same was true of methyl-d-galactosid. This glucosid was not attacked by the mycelium extract unless the substance had been present in the culture fluid. The apparent power of this organism to respond to the nutritional needs by producing new enzyms would be very striking if the author had demonstrated conclusively that all traces of lactase were absent from ordinary cultures.

TREHALASE.

The disaccharid trehalose was found in *A. niger* by Bourquelot, and its wide distribution in fungi was subsequently demonstrated by him. This substance is evidently a reserve carbohydrate stored up in the fungi, and before it can be utilized it must first be hydrolyzed into glucose. Accordingly Bourquelot examined the enzym preparation obtained from *A. niger* to see if it was capable of hydrolyzing trehalose. On adding the enzym solution to a solution of trehalose, the rotation decreased in a few hours, and the decrease continued until the sixth day, when the hydrolysis was complete, as was shown by the fact that the theoretical yield of glucose was obtained. According to Bourquelot, trehalase may be distinguished from maltase by the much lower temperature at which it is destroyed. Trehalase loses its activity at 64° C., while maltase remains active until a temperature of 74° C. is reached.

Theoretically, trehalose should be the food par excellence for this organism, as it is stored up for that purpose. But Bourquelot has shown that spores of *A. niger* germinate very slowly on solutions of trehalose. This fact he explains on the ground that only those enzyms are produced during germination which act on the food material contained within the spore. Later on another category of enzyms is produced, by the aid of which the organism can utilize the substances which it has stored up during a previous period of growth.

GENTIANASE AND GENTIOBIASE.

Gentianose, like raffinose, is a trisaccharid, and according to Bourquelot would require two distinct enzyms for its complete hydrolysis. Top yeast accomplishes the first stage of the reaction but carries it no further. Emulsin of almonds then acts on the disaccharid formed and completes the hydrolysis. Bourquelot found that *A. niger*, when grown upon Raulin's fluid produces both enzyms, as a complete hydrolysis of the gentianose occurred, yielding three molecules of hexose.

MELIZITASE.

Melizitose is another trisaccharid that would require two enzyms for its complete hydrolysis. The first stage is the cleavage into dextrose and turanose. The latter may then be still further decomposed by the action of dilute acids into two molecules of dextrose. Bourquelot and Hérissey found that only the first stage of this hydrolysis can be accomplished by *A. niger*. A 2.5 per cent melizitose solution mixed with an equal volume of the *Aspergillus* extract and allowed to digest for four days gave a decrease in rotation and a reduction of Fehling's solution, but the hydrolysis was not complete.

OTHER ENZYMS FOUND.

ZYMASE.

Among the molds there are transitional forms varying from the strictly aerobic type to those which readily adapt themselves to anaerobic conditions. *Penicillium* and *Aspergillus* belong to the former class, but even here we have quantitative differences. According to Duclaux, *Aspergillus* differs from *Penicillium* in that it can support a lack of oxygen for a longer time and with less injurious effects. It can even adapt itself for a certain length of time to an almost total deprivation of oxygen. When the organism is cultivated under partially anaerobic conditions, a small amount of alcohol may be detected in the medium. In the case of *P. glaucum* the amount of alcohol thus formed rarely exceeds one-thousandth of the total volume of the medium. This rapidly disappears again when access of air is restored.

The most successful experiments along this line are those of Kostytschew. This author first cultivated *A. niger* on a modified Raulin medium in which the cane sugar was replaced by dextrose. The rate of production of carbon dioxid was studied both with air access and after the air had been replaced by nitrogen. A marked diminution in the formation of carbon dioxid occurred after excluding the air. In a second series of experiments a culture of this organism three days old was sunk to the bottom of the flask by means of sterile glass beads. The air was then replaced by nitrogen and the flask sealed. After fourteen days in the thermostat the culture fluid was analyzed. The dextrose had decreased from 10.3296 grams to 10.0043 grams, while 0.1563 grams carbon dioxid and 0.1420 grams alcohol had been formed. The ratio between these two products is practically the same as that in the fermentation of sugar by yeast. The loss of sugar not represented by the carbon dioxid and alcohol the author ascribes to the formation of oxalic acid. From these experiments it is clear that the anaerobic respiration of *A. niger* takes on the character of an alcoholic fermentation when the mycelium is completely submerged. When the surface of the culture is exposed to an inert gas, the amount of carbon dioxid formed is insignificant.

Shortly after the publication of Kostytschew's paper, Junitzky proved that the anaerobic formation of carbon dioxid and alcohol by *A. niger* was due to an enzym closely resembling if not identical with the zymase of yeast. He grew the mold on Raulin's fluid until fructification began, then he ground the mycelium with sand and pressed out the juice in a Buchner press with 300 atmospheres. A solution of glucose was added to this juice, and the amount of carbon dioxid and alcohol was determined after twenty-four hours. Although only a small percentage of the sugar was fermented and a large amount of enzym had to be used, the presence of a zymase was demonstrated. This zymase was formed by the mold during its ordinary aerobic vegetative processes.

OXIDASE.

Molds do not produce the characteristic oxidizing enzyms to anything like the extent to which such enzyms are found in many of the higher fungi. Bourquelot, who studied the oxidase and laccase of mushrooms, as well as many enzyms of *A. niger* and *P. glaucum*, reports no observation whatever of the occurrence of any oxidizing enzyms in these lower fungi. Shibata, in his study of the amidases, failed to find any tyrosinase in *A. niger*. Yet it has long been known that the common molds grow normally only in the presence of oxygen and that the medium must contain an oxidizable substance. As the ultimate products are principally water and carbon dioxid, we must assume that an oxidation takes place.

Herzog and Meier studied the effect of cultures of *P. glaucum* upon various hydroxy acids in the medium. Cultures were made upon diluted beer wort, and when the rate of production of carbon dioxid had become constant they added a solution of the hydroxy acid and determined the increased output of carbon dioxid as well as the amount of acid consumed. A fairly constant ratio was found between the increase in carbon dioxid and the disappearance of the acid. Lactic, tartaric, malic, mandelic, and beta-hydroxybutyric acids were readily oxidized, while glycolic, citric, pyroracemic, and hydroxyisobutyric acids showed no appreciable oxidation. The presence of an asymmetric carbon atom is therefore necessary. On killing the mold with acetone or methyl alcohol and quickly drying, the authors obtained a powder which likewise liberated carbon dioxid from hydroxy acids.

In a second paper the same authors showed that the optical antipodes of hydroxy acids are oxidized at different rates. This explains the separation of racemic mixtures by cultures of fungi, first observed by Pasteur. An enzym prepared by treating the mycelium with liquid air was found to liberate carbon dioxid from lactic acid. The authors call the enzym "acidoxydase."

Part of this carbon dioxid undoubtedly comes from the carboxyl group of the acid. In the case of cinnamic acid this type of reaction has been demonstrated. Oliviero showed that *A. niger* and *P. glaucum* transform cinnamic acid into styrol. The organisms were grown on Raulin's fluid until mature, then shaken violently to liberate the enzym and filtered through a Chamberland filter. On the addition of small quantities of sodium cinnamate the characteristic odor of styrol was noticed. Herzog and Ripke confirmed this observation by growing *P. glaucum* upon a large amount of beer wort to which ammonium cinnamate had been added. In three days the characteristic odor of styrol was noticed, and the substance was isolated and identified.

SUMMARY OF THE LITERATURE.

A considerable number of enzyms have been found by previous investigators in species of *Penicillium* and *Aspergillus*. Those which have been shown to act in the absence of living cells are: Protease, nuclease, lipase, emulsin, amylase, inulase, raffinase, sucrase, maltase, trehalase, gentianase, melizitase, amidase, zymase, and oxidase. Some of these occur in very active form; others are more difficult to demonstrate on account of the small amount present. Lactase is said to occur only when lactose is the source of carbon in the culture medium. The nature of the protease has not been established, though most of the authors consider it as closely resembling trypsin.

EXPERIMENTAL WORK.

CHEMICAL DIFFERENCES IN SPECIES OF PENICILLIUM.

The résumé of literature to which the preceding pages have been devoted comprises both the intracellular and extracellular enzyms of *Aspergillus* and *Penicillium*. It will be seen that with very few exceptions previous work has been confined to two species—*A. niger* and *P. glaucum*. This is particularly true of those investigations which deal primarily with the intracellular enzyms. Cultures of these organisms are easy to obtain, as they make their appearance spontaneously upon almost any kind of substratum that contains a carbohydrate. Many of the cultures for enzym experiments were undoubtedly obtained in this way; for some authors admit having made transfers from media that had accidentally become contaminated with these molds, and it is of course impossible to say with what care the organisms were identified. The fact that binomial nomenclature is used leaves it to be inferred that the cultural aspects and morphology were found to correspond with the original description. Assuming, then, that this agreement was found, the results are comparable provided the original description is sufficiently accurate to include only a single species. As far as *A. niger* is concerned this is probably true. Van Tieghem's description of this organism is accurate and minute, and moreover there are very few organisms which might possibly be mistaken for *A. niger.* On the other hand, Link's description of *P. glaucum* is so vague as to include all green Penicillia. In fact the designation "*P. glaucum*" has almost come to be synonymous with "green mold."

The question now arises, do all species of *Penicillium* show the same physiological and chemical characteristics? A detailed discussion of these fungi from the standpoint of taxonomy would of course be out of place in this paper. Suffice it to say that there are a number of green Penicillia, all of which answer to Link's description of *P. glaucum,* but which show marked chemical differences under definite conditions. In a monograph by Dr. Charles Thom[a] some half dozen commonly occurring green Penicillia are described. Cultural studies of these organisms show that some liquefy gelatin and some do not; some secrete a red or yellow dye into the medium while others do not; and some give a characteristic odor which others fail to produce. Morphological differences are likewise to be found. It is therefore not improbable that differences in enzymatic activity may be equally pronounced. While there is some controversy as to whether it is justifiable to distinguish species on purely physiological grounds, yet in chemical experiments these chemical differences should be taken into account. Many of the conflicting statements in the literature, especially those concerning the proteolytic enzyms, may be explained on the assumption that different organisms were used under the same name. If species so closely related show decided chemical differences, still greater variations may be expected where morphological differences are more pronounced.

THE ORGANISM SELECTED, PENICILLIUM CAMEMBERTI.

In selecting a *Penicillium* for enzym experiments the writer has endeavored to use one whose identity is fully established, and to obtain pure cultures from a reliable source. For a number of reasons *P. camemberti* was considered particularly well adapted for these experiments. The cultures were obtained from Dr. Charles Thom, of the United States Department of Agriculture, by whom the fungus was first described. The organism in question is of considerable economic importance because of its association with the cheese industry. It is the principal factor in the ripening of the well-known Camembert type of soft cheese. The manner in which it produces the ripening will be discussed in connection with the proteolytic enzym. So intimately is this mold associated with Camembert cheese and so readily is it recognized that it is not a difficult matter to obtain a pure culture by simply making a transfer from a Camembert cheese purchased in the market. This mold possesses the additional advantage of thriving admirably under artificial conditions and producing a dense mycelium rich in enzyms.

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[a] Thom, Charles. Cultural studies of Penicillium. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 118. 1910.

CHOICE OF A CULTURE MEDIUM.

Having secured the organism in pure culture, a number of experiments were made in order to determine what medium was most suitable for propagating it. Obviously, if the mycelium of the mold is to be prepared in the form of a dry powder as free as possible from nutrient material, a solid medium such as gelatin or agar is undesirable. All of the fluid media described by Raulin, Wehmer, Czapek, Dean, and a host of others consist essentially of a carbohydrate solution to which a few inorganic salts have been added. Raulin's fluid is particularly well adapted to *A. niger*, but it has been used a great deal for other species also.

The choice of a medium for *P. camemberti* was therefore carefully considered at the outset. Inoculations were made upon most of the media described in the literature, and while the organism seemed to thrive upon all of them, the media which gave the most luxuriant growth were those of Raulin and Czapek, sodium nitrate being added to the latter as the source of nitrogen. Where cultural studies are to be made to determine the effect of nutrition on the secretion of enzyms other carbohydrates may be substituted for the cane sugar. For such experiments Raulin's fluid is objectionable, as it contains enough free acid to decompose some of the more easily hydrolyzable carbohydrates during sterilization. In Czapek's fluid the acidity is due entirely to acid phosphates, and experiments showed that raffinose, inulin, etc., could be sterilized in this medium without undergoing decomposition. This medium was therefore adopted for the sake of uniformity. Its composition is as follows:

Water	1,000.0
Magnesium sulphate (cryst.)	.5
Potassium acid phosphate	1.0
Potassium chlorid	.5
Ferrous sulphate	.01
Sodium nitrate	2.0
Cane sugar	30.0

METHODS OF CULTURE.

The above-described medium was placed in a large number of Erlenmeyer flasks to the depth of about 3 cm. The mouth of each flask was then closed with a cotton plug and the flasks were sterilized in an autoclave at 110° C. for half an hour. Occasionally a slight cloudiness appeared in the medium after sterilization, due probably to the precipitation of magnesium phosphate, but this disappeared after the mold had begun to grow. Inoculations were made from a pure culture by means of a sterile platinum wire. The cultures were allowed to develop in diffuse daylight at laboratory temperature. In about three days white specks were seen floating on the surface of the fluid, and these rapidly spread until the whole surface became covered with a dense white mycelium. The mycelium continued to develop and increase in thickness until about the tenth day, when the bluish-green color of the spores began to appear.

PREPARATION OF ENZYM POWDER.

Experiments showed that the intracellular enzyms are present in greatest amount just before the fruiting stage is reached. By this time the sugar, which was completely inverted some days previously, is nearly used up. The reaction, which was at first acid, is now neutral or slightly alkaline to litmus. If now the culture is allowed to go on still further and the surface of the colony to become covered with spores, the enzyms are rapidly liberated into the medium and the mycelium is found to be less active.

Just as the colored spores began to appear, therefore, the mold was removed and treated according to Albert and Buchner's method for preparing "acetondauerhefe." The method as adapted to these experiments is as follows: The whole mycelium, which can be removed from the flask in the form of a thick disk, is immersed for a few moments in running water, then squeezed to remove as much as possible of the water, and run through a hashing machine. The wet mass thus obtained is immersed for ten minutes in a large volume of acetone, with constant stirring; then it is filtered off with suction. It is now immersed again for two minutes in a fresh quantity of acetone and filtered as before. The third time it is immersed in ether for three minutes, sucked as dry as possible, and spread out in a thin layer for several hours until the odor of ether is no longer perceptible. A coarse dry powder results, which is ground still finer by a suitable grinding machine.

COMPOSITION OF ENZYM POWDER.

This powder contains, besides the enzyms, all the constituents of the cells that are not soluble in acetone and ether. Needless to say, the osmotic pressure produced within the cells by this treatment ruptures the cell walls, so that the enzyms can easily be extracted by subsequent treatment with water. The lipoids, however, have been removed almost completely by the acetone and ether. The acetone from the first extraction has a bright yellow color and when distilled until aqueous vapors begin to pass over yields a flocculent precipitate which has an odor like that of some of the terpenes. The extracted powder is white or greenish in color and is practically anhydrous. It easily becomes electrified so that the particles tend to fly apart. It contains 4.50 per cent ash and 4.35 per cent nitrogen. The greater part of the nitrogen is not in the form of true protein, but consists of chitin or nitrogenous cellulose. About 1 per cent of nitrogen can be extracted by water. The aqueous extract has an opalescent appearance, and gives the biuret, Millon, and Hopkins-Cole reactions, though rather feebly.

EXPERIMENTAL METHODS.

In all the experiments about to be described, unless otherwise indicated, this powder was used either in suspension or in the form of an aqueous extract as the source of enzym. The advantage of this method is that a preparation of uniform activity can be used in a whole series of experiments. The powder when kept out of the light and in well-stoppered bottles seems to retain its activity indefinitely. A specimen more than a year old is apparently as active as it was when freshly prepared.

The digestions were all carried out in a thermostat maintained at a temperature of 35 to 37° C. Two to 5 per cent of toluene, depending upon the amount of surface exposed, was used as an antiseptic, unless otherwise indicated. All the tubes and flasks were tightly stoppered to prevent any change in volume by evaporation and any loss of toluene. Whenever aliquot portions were taken for analysis the mixture was first cooled down to laboratory temperature. The time of digestion varies in the different experiments, but is stated in each case. Control experiments were usually made, either with a mixture of zymolyte and boiled enzym, or else with zymolyte and unboiled enzym separately.

ENZYM STUDIES.

PROTEASE.

Extracellular protease, cheese ripening.

The economic importance of *Penicillium camemberti* depends upon its proteolytic enzym. In the manufacture of Camembert cheese the mold makes its appearance on the surface of the cheese soon after its removal to the ripening room. The mold continues to develop, and about the time the fruiting stage is reached a softening of the curd appears just under the mycelium. This softened layer rapidly increases in depth until in four or five weeks the hard curd has assumed a soft, waxy texture throughout. The change is evidently due to an enzym secreted by the mold and liberated into the curd mass. Investigations by the writer[a] have shown that this change in the physical appearance of the curd is accompanied by chemical changes which consist in the conversion of the insoluble paracasein into soluble proteoses and peptones and amino acids.

Intracellular protease.

The proteolytic enzym is also contained in the mold powder in the intracellular form, and its presence can readily be demonstrated. As will be seen shortly, the formation of protease is independent of the presence of protein in the culture medium. It occurs when the mold is grown upon sugar and sodium nitrate, as well as when gelatin or peptone have furnished the nitrogen. The proteolytic activity of the powder obtained as described above was tested with a number of proteins. As the soluble protein of the mold itself gives the biuret reaction and undergoes autolysis with the formation of tryptophan, these reactions could not be relied upon to show positively whether the added protein had actually been digested. The change in solubility of the protein was therefore taken as the criterion.

The first tests were made with the two proteins most commonly used in demonstrating both peptic and tryptic enzyms, namely, coagulated egg white and fibrin. In these experiments, as well as in those following, an enzym solution was prepared by extracting the mold powder for two hours with twenty times its weight of water.

Experiment 1.—Ten cubic centimeters of the filtered extract were placed in each of three small Erlenmeyer flasks. To the first was added 1 c. c. of 1 per cent HCl, to the second 1 c. c. of 1 per cent Na_2CO_3 solution, and to the third 1 c. c. water. In each of the flasks were placed two Mett tubes of coagulated egg white. At the end of a week the tubes were examined and none of them showed any signs whatever of digestion.

Experiment 2.—The above experiment was repeated, using a small flock of fresh fibrin in place of the Mett tubes. In about three days the fibrin in the flask containing alkali showed signs of disintegration, and at the end of a week it had completely disappeared. The fibrin in the neutral medium also showed some solution, though not quite so marked. The acid fibrin was least altered. This evidence of digestion of fibrin by the mold extract is, however, offset by the fact that the fibrin in the control flasks with boiled enzym behaved in the same way, though the rate of digestion was somewhat slower, especially in the neutral flask. The disappearance of the fibrin was probably caused in part at least by leucocyte enzyms present in the fibrin.

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[a] Dox, Arthur W. Proteolytic changes in the ripening of Camembert cheese. U. S. Department of Agriculture, Bureau of Animal Industry, Bulletin 109. 1908.

Experiment 3.—The behavior of the mold extract toward boiled fibrin was then tested. The experiment was conducted exactly as described above, except that boiled fibrin was substituted for the fresh fibrin. At the end of a week the fibrin in all of the flasks was apparently unaltered. In the presence of acid a swelling had occurred, but no signs of disintegration.

Experiment 4.—The colorimetric method recently described by Roaf[a] was also tried. This method makes use of fibrin that has been stained with Congo red and the dye rendered permanent by immersing the fibrin in boiling water. A liberation of color from fibrin carefully prepared in this manner indicates proteolysis. Ten cubic centimeters of the mold extract were used, as before, in acid, alkaline, and neutral solution, and approximately equal amounts of stained fibrin. After five days only a very slight amount of color was liberated, the intensity of which was about the same as that in the corresponding control tests.

Experiment 5.—A number of other proteins were tested with respect to their digestibility by the mold enzym. The same amount of extract was used, and the tests were carried out in acid, alkaline, and neutral media. At the end of a week the acid or alkali that had been added was exactly neutralized by an equivalent amount of alkali or acid, and in the case of the globulins the precipitation was aided by heating. The protein was then filtered on a tarred filter paper, dried at 110° C., and weighed. The control experiments are given here only where they show a decided difference from the others.

Protein tests in neutral, acid, and alkaline media containing mold extract.

Protein added, 0.1 gram.	Protein recovered.		
	Neutral.	N/20 HCl.	N/20 Na_2, CO_3.
Zein	0.0998	0.0996	0.0987
Cryst. edestin	.0989	.0993	.0987
Excelsin	.0990	.0991	.0970
Elastin	.0997	.1002	.0980
Collagen	.0991	.0996	.1005
Ovovitellin	.0978	.0982	.0974
Coconut protein	.0991	.0982	.0990
Casein	.0006	.0876	.0323
Casein (control)	.0994	.0980	.1003

Of the eight proteins tested, casein is the only one that was appreciably digested. The activity of the enzym toward this protein is greatest in the very faint natural acidity of the extract. The digestion is less rapid in the presence of sodium carbonate and is almost inhibited by hydrochloric acid of the strength used. In the former case the weakening of the enzym probably occurred before the alkali had combined with the casein, because "nutrose," the sodium salt of commerce, was found to digest readily. The readiness with which the enzym digests casein is not surprising, in view of the fact that this protein forms the natural habitat of the organism, and is so easily digested by the living mycelium.

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[a] Roaf, H. E. A new colorimetric method to show the activity of either ' 'peptic' or "tryptic" enzyms. Biochemical Journal, vol. 3, pp. 188–192. Liverpool, 1908.

Experiment 6.—As the living organism has also the power of growing on gelatin and liquefying it, the enzym might be expected to act in the same way. The effect of the enzym solution on this albuminoid was tested by Fermi's method with liquid gelatin.[a]

In each of three tubes 5 c. c. of 20 per cent gelatin containing thymol were placed. The jelly was liquefied by warming to 35° C., then 5 c. c. of enzym solution were added, and 5 c. c. of water. In one tube the mixture was made acid by the addition of hydrochloric acid to 0.1 per cent, to another sodium carbonate was added to 0.1 per cent, and the third was made neutral. Control tubes were prepared in the same way by adding boiled enzym. The mixtures were left in the thermostat at 35° C. for twenty-four hours. They were then removed and cooled down to 15° C. by immersion in running water. The contents of the tubes to which the unboiled enzym had been added remained liquid, with the exception of the acid gelatin, while those containing boiled enzym quickly solidified to a stiff jelly.

Experiment 7.—The action of the enzym on gelatin may be demonstrated with equal readiness by Fermi's method with tubes of solid gelatin.

To each of three small test tubes 2 c. c. of 10 per cent gelatin containing 0.2 per cent thymol were added. One was made 0.1 per cent acid with hydrochloric, another 0.1 per cent alkaline with sodium carbonate, and the third was kept neutral. Two cubic centimeters of a 3 per cent mold extract were then added to each, and the same amount of acid or alkali as the gelatin contained. A few drops of toluene were added and the tubes left at room temperature. Every twenty-four hours the level of the gelatin was marked on a strip of paper pasted on the tube. The acid gelatin showed no signs of digestion; in fact, there was a slight swelling above the mark indicating the original level. In the other tubes the linear liquefaction was as follows:

Hours.	Neutral.	Alkaline.	Acid.
	Mm.	*Mm.*	*Mm.*
24	4	1	....
48	7	2	....
72	9	2	....
96	10	2	....

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[a] Fermi, C. Reagentien und Versuchsmethoden zum Studium der proteolytischen und gelatinolytischen Enzyme. Archive für Hygiene, vol. 55, pp. 140–205. München, 1906.

Experiment 8.—It has already been shown that the enzym does not act on coagulated egg white in Mett tubes. A solution of crystallized egg albumin was tested in order to see if different results might be obtained.

Ten cubic centimeters of the enzym solution were used and 2 c. c. of a solution of crystallized ovalbumin. After remaining four days in the thermostat the solutions were made just faintly acid and the unchanged albumin was coagulated by boiling. This precipitate was filtered and washed, and the nitrogen determined by the Kjeldahl method.

Enzym.	Coagulum (nitrogen).		
	Neutral.	0.1 per cent HCl.	0.1 per cent Na_2CO_3.
	Grams.	*Grams.*	*Grams.*
Unboiled	0.0339	0.0357	0.0348
Boiled	.0344	.0359	.0358

Neither coagulated egg white nor soluble ovalbumin undergoes any appreciable digestion.

Experiment 9.—Casein, as has been seen, is readily digested by this enzym. In order to determine to what extent the digestion goes on, the following experiment was carried out: Ten grams of casein "nach Hammarsten" were suspended in 200 c. c. water. Five grams of mold powder were extracted for two hours with 100 c. c. water, then filtered and added to the casein suspension. The bottle containing this mixture was placed in a bath maintained at a temperature of 40° C. and shaken at frequent intervals during the day. In about two days the casein had begun to disappear. In a week it had all disappeared with the exception of a scum which remained in the layer of toluene at the surface. This scum was filtered off, dried at 100° C., and weighed. Eight-tenths of a gram was obtained and the phosphorus content was found to be 0.76 per cent. It was probably unaltered casein containing adsorbed impurities from the enzym solution. The filtrate, which was yellow in color, was left in the thermostat until the digestion had gone on for thirty-two days. At the end of that time the total nitrogen, the nitrogen in the filtrate after precipitating with tannic acid, and the nitrogen as ammonia were determined, also the total and inorganic phosphorus. The difference between the first two determinations represents caseoses and peptones; that between the second and third represents amino acids.

Test to determine digestion of casein.

[10 grams casein, 200 c. c. water, 100 c. c. 5 per cent mold extract.]

Days.	Nitrogen as—	In 10 c. c.	Nitrogen calculated as—	In 10 c. c.
		Grams.		*Grams.*
0	Total nitrogen	0.0036		
32	Total nitrogen	.0444	Proteoses and peptones	0.0105
32	Tannin filtrate	.0339	Amino acids	.0312
32	Ammonia	.0027	Ammonia	.0027
55	Total nitrogen	.0444	Proteoses and peptones	.0060
55	Tannin filtrate	.0384	Amino acids	.0357
55	Ammonia	.0027	Ammonia	.0027
32	Total phosphorus in 50 c. c., 0.0120 gram.			
32	Inorganic phosphorus in 50 c. c., 0.0124 gram.			

The digestion of casein goes beyond the peptone stage, the principal products being amino acids. The solution gave a pink biuret reaction and a strong tryptophan reaction with bromine water. The material remaining after the analytical determinations had been made was evaporated to a sirup and allowed to stand in a cool place for several days. Characteristic crystals of tyrosin and "leucin" separated out, and these were identified under the microscope.

Experiment 10.—The proteoses and peptones which are formed as intermediary products in the digestion of casein are rapidly broken down into simpler products. Although fibrin is not acted upon by this enzym, it was thought that the proteoses and peptones derived from fibrin might be attacked more readily. The following experiment was therefore made: Three grams of "Witte peptone" were dissolved in 50 c. c. water; 2 grams of mold powder were extracted for two hours with 50 c. c. water, filtered, and added to the peptone solution. After remaining in the thermostat for fourteen days, nitrogen determinations were made as in the preceding experiment.

Test to determine digestion of peptone.

[3 grams Witte peptone, 50 c. c. water, 50 c. c. enzym, 14 days.]

Nitrogen as—	In 10 c. c.	Nitrogen calculated as—	In 10 c. c.
Witte peptone solution:	*Grams.*	Witte peptone solution:	*Grams.*
Total nitrogen	0.0444	Proteoses and peptones	0.0099
Tannin filtrate	.0345	Amino acids	.0320
Ammonia	.0025	Ammonia	.0025
Control—boiled enzym:		Control—boiled enzym:	
Total nitrogen	.0444	Proteoses and peptones	.0336
Tannin filtrate	.0108	Amino acids	.0096
Ammonia	.0012	Ammonia	.0012

Influence of acid and alkali.—In the experiments with Witte peptone, as well as in the casein experiment, no acid or alkali was added to the digestion mixture. The extract from the mold, however, was faintly acid to litmus, though neutral to methyl orange and dimethylaminoazobenzene. The acidity persisted after the digestion had gone on for some time. To determine the effect of acids and alkalies a series of tests was made. Different amounts of decinormal acids and alkalies were taken and the total volume made up to 10 c. c. with distilled water, then 0.1 gram Witte peptone was added and 0.1 gram mold powder. After twenty-four hours in the thermostat the tryptophan test was applied, using the same number of drops of bromine water in each case. With hydrochloric, lactic, and phosphoric acids the intensity of the color decreased with increasing concentration, until with N/20 acid the test was negative. Alkalies were much less inhibitory than acids. The reaction was still positive though quite faint when sodium carbonate or ammonia was present in decinormal concentration.

Summary.—From the experiments described above it will be seen that the intracellular protease of *Penicillium camemberti* readily digests casein, gelatin, and Witte peptone, but has no action on fibrin, ovalbumin, or other native proteins. In this respect it is closely analogous to the protease discovered by Cohnheim [a] in the intestines of mammals, and named by him "erepsin." Vines [b] has found erepsin in a large number of plants, and has been able to separate it from the peptic enzym which usually accompanies it. According to Vines, "the ereptase of plants differs from that of animals only in that its reaction range is more extensive in the direction of acidity, and is, perhaps, less extensive in the direction of alkalinity."

At the time the protease of molds was being investigated by Bourquelot and others, erepsin had not been discovered. These authors regarded the enzym as closely resembling if not identical with trypsin. There is a possibility that the organisms studied, especially *Aspergillus niger*, secretes a small amount of peptic or tryptic enzym, enough to account for the digestion obtained in some cases with fibrin; or it may be that the fibrin itself contained proteolytic enzyms from the leucocytes.

To determine whether the liquefaction of gelatin was due to a specific "glutinase," the writer prepared an extract from a dog's intestine and tested its action on gelatin by Fermi's method, with positive results. The digestion must have been due to erepsin, for the same extract had no action on fibrin.

NUCLEASE.

In Iwanoff's paper, which is the only one in the literature having to do with the enzym of molds which acts on nucleic acid, the author states that the organisms were cultivated on media containing nucleic acid. His results show no evidence that a mold will produce nuclease when it is cultivated upon a medium consisting entirely of carbohydrate and inorganic salts. The experiments described in the preceding pages have shown that the production of protease in *P. camemberti* is independent of the presence of protein in the culture medium. Whether nuclease is produced under similar conditions was an important point that remained to be investigated.

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[a] Cohnheim, O. Die Umwandlung des Eiweiss durch die Darmwand. Zeitschrift für physiologische Chemie, Band 33, p. 451–465. Strassburg, 1901.

[b] Vines, S. H. The proteases of plants. Annals of Botany, vol. 23, No. 89, p. 17. London, 1909.

A specimen of yeast nucleic acid was purified by dissolving and precipitating by alcohol. Five grams of the product were dissolved in just enough dilute potassium hydroxid to give a solution neutral to litmus. Two grams of mold powder were extracted for an hour with 100 c. c. water, filtered, and the solution was added to the nucleic-acid solution. The total volume was then made up to 250 c. c. and toluene added for an antiseptic. After forty-five days in the thermostat at 35 to 37° C. the solution was tested with ammoniacal silver nitrate and magnesium mixture. Both of these reagents gave a precipitate, while in the control experiment the precipitates obtained were almost negligible. One hundred cubic centimeters of the solution were taken for the purin determination. The purins were precipitated by copper sulphate and sodium bisulphite, washed with hot water, and decomposed by hydrogen sulphid. After boiling off the excess of hydrogen sulphid the filtrate was reprecipitated by copper sulphate and sodium bisulphite. The amount of precipitate was not large enough for a separation of the purins, so the nitrogen contained in it was determined by the Kjeldahl method. Total phosphorus and phosphorus as phosphoric acid were determined in 50 c. c. portions. The results are expressed as grams per 100 c. c.:

	Gram.
Total nitrogen	0.1788
Purin nitrogen	.0663
Total phosphorus	.0859
Phosphorus liberated	.0512
Control—boiled enzym:	
Total nitrogen	.1788
Purin nitrogen	.0036
Total phosphorus	.0859
Phosphorus liberated	.0041

There can be no doubt that the decomposition of the nucleic acid was due to an enzym. And it is evident that the nuclease of *P. camemberti* is formed irrespective of the presence of nucleic acid in the culture medium. The decomposition is more complete than that obtained by Iwanoff, owing to the greater length of time through which the enzym acted, and probably also to a greater activity of the enzym preparation. Iwanoff's enzym failed to liquefy gelatin, although at least one of the organisms used by him—*A. niger*—has been shown by other investigators to contain a gelatin-liquefying enzym. These investigations, however, are not strictly comparable with those of Iwanoff, because of the different methods of culture and the different conditions of the experiments. It will be noted that the ratio between the purin nitrogen and the phosphorus liberated is different from that obtained by Iwanoff with thymonucleic acid (see p. 21).

AMIDASE.

It was seen in the experiments under protease that the mold powder had the power of liberating ammonia from casein and Witte peptone. This effect might be attributed to a deamidizing enzym. The liberation of ammonia from the protein molecule during proteolysis is probably due to a breaking down of such amids as asparagin and glutamin, which are intermediary products, into ammonia and aspartic and glutaminic acids. If this is the case, the same enzym might be expected to hydrolyze other amids in a similar manner.

In the following experiments the enzym preparation from the sucrose medium was tested with several amino compounds. A weighed quantity (0.2 gram) of the substance was dissolved or suspended in 20 c. c. water, and 0.5 gram mold powder added. Toluene was used for an antiseptic. After several days in the thermostat the contents of the flasks were transferred to cylinders and the ammonia determined by Folin's method. The following figures were obtained:

Test to determine the decomposition of amids.

Substance.	Formula.	Days.	$N/5H_2SO_4$.	Control.	N as NH_3.
			c. c.	*c. c.*	*Gram.*
Urea	$\diagup NH_2$ CO $\diagdown NH_2$	4	6.3	0.5	0.0174
Asparagin	$CO-NH_2$ $HOOC-CH_2-CH-NH_2$	4	5.0	.2	.0144
Biuret	$\diagup NH_2$ CO $\diagdown NH$ $\diagup$ CO $\diagdown NH_2$	5	.4	.2	.0006
Acetanilid	C_6H_5 $\diagdown NH$ $CH_3CO \diagup$	5	.8	.8	.0000
Alanin	CH_3CH-NH_2 $HOOC$	5	.7	.2	.0015
Benzamid	$C_6H_5CO-NH_2$	5	.9	.2	.0021

Urea and asparagin are both readily acted upon. Benzamid and alanin are hydrolyzed to a considerably less extent, while the hydrolysis with biuret is doubtful.

Hippuric-acid-splitting enzym.—The mold powder was found to have the power of hydrolyzing hippuric acid into benzoic acid and glycocoll (aminoacetic acid). The experiment was conducted as follows: Two-tenths of a gram of hippuric acid was dissolved in just enough sodium hydroxid to give a neutral reaction with litmus. The volume was made up to 20 c. c. and then 0.4 gram mold powder added. At the end of a week the mixture was filtered and the calculated amount of sulphuric acid added to combine with the sodium. This solution was shaken several times with petroleum ether in a separatory funnel, and the petroleum allowed to evaporate. A crystalline residue was left which after recrystallization from water melted at 121° C. and had the crystalline appearance of benzoic acid. In the control experiment with boiled enzym no residue was obtained on evaporating the petroleum. On repeating the experiment and extracting first with ethyl acetate according to the method of Bunge and Schmiedeberg the weight of the benzoic acid obtained was 0.1085 gram. As 0.2 gram hippuric acid yields 0.148 gram benzoic acid on complete hydrolysis, the hydrolysis in this case is 76 per cent.

The effect of cultivating the organism on media containing amino compounds would probably be an increase in the amount of amidase produced by the mycelium. This point, however, still remains to be determined. So far as the influence of nutrition on the production of enzym has been studied in the case of the carbohydrate-splitting enzyms, as will be pointed out later, the effect has been to increase the production of the specific enzym necessary for the hydrolysis of the given subtratum. There is no reason why the same relation between subtratum and enzym formation should not hold true also for other enzyms such as amidase.

LIPASE.

The lipolytic activity of the enzym powder prepared from cultures of *P. camemberti* that had been grown on cane sugar was very feeble. This might be expected in view of the small amount of lipase obtained by other investigators who worked with *P. glaucum* and *A. niger*. By carefully titrating the acid formed after the enzym had been allowed to act upon the ester, a slight lipolytic activity could be demonstrated. In these experiments an extract of the mold powder, prepared as already indicated, was used. The extract alone showed no appreciable increase in acidity after remaining in the thermostat throughout the experiment. Ten cubic centimeters of the enzym solution were diluted with an equal volume of water and 0.2 c. c. of the ester added. A few drops of toluene were added for an antiseptic, and the mixture was left in the thermostat for three days. The solution was then titrated with decinormal sodium hydroxid, using phenolphthalein as an indicator.

Ester.	N/10NaOH.	Control.
	c. c.	*c. c.*
Triacetin	1.6	1.0
Monobutyrin	1.8	1.0
Amyl acetate	1.4	1.2
Ethyl butyrate	2.0	1.3
Ethyl acetate	1.4	1.1

In every instance there is a slight increase in acidity, though in some cases it is only very slight indeed. Glycerin monobutyrin and ethyl butyrate gave the greatest increase, while with amyl acetate and ethyl acetate the increase is much smaller.

The very slight extent to which the esters were hydrolyzed may be explained in two ways—there may be a loss of enzym during the process of preparation, or it may be that the culture medium was unsuitable for the production of lipase. It has been claimed that during the extraction of fat from seeds a considerable part of the lipase goes over into the ether extract. According to Taylor[a] lipase is insoluble in ether, but is soluble in ether containing a fatty body. If such is the case, part of the lipase may be lost when the mycelium of the mold is treated with acetone and ether. Again, the lack of lipase may be due to the fact that the medium contained no ester to stimulate the formation of the enzym. As will be seen in subsequent experiments with other enzyms, there seems to be a greater formation of a specific enzym when its particular zymolyte is present in the medium. By analogy, the production of lipase would be increased by cultivating the mold on a medium containing a fat or an ester.

Cultures of this organism do have the power of hydrolyzing fat under proper conditions. An experiment was made in which a suspension of pure butterfat in a solution of inorganic salts was inoculated with *P. camemberti.* The mold grew very slowly at first, but after a time the growth appeared to be normal. The butterfat changed its appearance completely, and a white mass of minute crystals was formed.

Further experiments will be conducted to determine whether the fresh mycelium is more active than the "dauer" preparation and whether the presence of fat in the medium increases the amount of lipase formed.

EMULSIN.

Just as the four enzyms already discussed are formed in the absence of their respective zymolytes from the culture medium, so emulsin is formed in the absence of glucosids. The same powder that was used in the preceding experiments was tested with respect to its glucosid-splitting power. The following glucosids were used: Amygdalin, arbutin, salicin, and phlorhizin. Two-tenths of a gram of the glucosid was dissolved in 20 c. c. water, and 0.1 gram mold powder was added. After three days in the thermostat the mold powder was filtered off and the solution tested to see if hydrolysis had occurred.

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[a] Taylor, E. A. On the action of lipase. Journal of Biological Chemistry, vol. 2, No. 1, p. 87. New York, 1906.

Amygdalin, when completely hydrolyzed, yields dextrose, benzaldehyde, and hydrocyanic acid. All three of these products were demonstrated in the digestion mixture. A strong odor of bitter almonds at once revealed the presence of benzaldehyde. The presence of free hydrocyanic acid was shown by distilling a portion of the liquid from a test tube and testing the distillate with ferrous sulphate and ferric chlorid, whereupon a precipitate of Prussian blue was obtained. The dextrose was identified by its osazone.

Arbutin hydrolyzes into dextrose and hydroquinone. In the presence of the enzym the solution rapidly darkened in color on account of the oxidation of the hydroquinone. When tested with Fehling's solution black oxidation products were formed, as well as a heavy precipitate of cuprous oxid.

Salicin breaks up into o-hydroxybenzylalcohol and dextrose. The former was recognized in the digestion solution by the deep violet color it gave with ferric chlorid. The dextrose was identified as before, by the formation of insoluble osazone.

Phlorhizin when hydrolyzed yields dextrose and phloretin, and the latter on further hydrolysis gives phloretic acid and phloroglucinol. After the enzym had been allowed to act on this glucosid a reduction of Fehling's solution was obtained amounting to 0.0170 gram cuprous oxid for 0.2 gram of the glucosid. Only a partial hydrolysis was obtained in this case. The digestion mixture did not give a color with ferric chlorid, showing that there was no liberation of phloroglucinol.

From the above experiments it will be seen that three typical glucosids—amygdalin, salicin, and arbutin—are readily decomposed by an intracellular enzym produced when the mold is cultivated on a medium containing only a carbohydrate as the source of carbon. Phlorhizin is also hydrolyzed, though less readily and less completely.

ENZYMS ACTING ON CARBOHYDRATES.

The carbohydrate-splitting enzyms present in extracts or suspensions of the mold can readily be demonstrated by several methods. The first consists in observing the change in optical rotation of the carbohydrate solution to which the enzym had been added. Another method depends upon the increase in reducing power as determined by Fehling's solution. As only two of the ordinary hexose anhydrids—lactose and maltose—reduce Fehling's solution before inversion, this test may be applied qualitatively. The third method depends upon the formation of an osazone separating from the hot solution with a characteristic appearance under the microscope. When this method is applied to solutions containing the more complex polysaccharids, such as starch, dextrin, and inulin, the latter must first be removed from the solution by precipitation with alcohol. The first two of these methods give very accurate results and may be used for quantitative work. The osazone method, however, is merely qualitative and may even give negative results where only a small proportion of the sugar has been inverted.

In all the enzym experiments in which the hydrolysis is measured by sugar determinations a slight correction must be made for a reducing sugar formed by the autolysis of the mold. It was found that 0.1 gram of the mold powder suspended in water and kept at thermostat temperature for three days gave 0.0060 gram cuprous oxid, while the control with boiled enzym gave no reduction at all. A number of similar experiments showed that the amount of reducing sugar formed in this way was fairly constant. The sugar was found to yield an osazone separating from the hot liquid and having the crystalline appearance and melting point of phenylglucosazone. It is not unlikely that this reducing sugar has been a source of error in the work of previous investigators.

By using a small amount of powder, say 0.1 gram, the error is reduced to a minimum. It is very small in proportion to the amount of reducing sugar formed from the added carbohydrate, and it has no appreciable effect upon the optical rotation. In most of the determinations the amount 0.0060 gram is simply deducted from the weight of the cuprous oxid obtained. This slight error may be eliminated entirely, as was done in the case of one or two experiments, by subjecting the autolyzing mold extract to dialysis in a collodion bag until the sugar formed had disappeared. This procedure seemed to weaken the enzym to some extent, and this is very likely due to a destructive action of the proteolytic enzym, for according to Vandervelde[a] most enzyms do not pass through cellulose membranes. When the fresh mold or a preparation dried in the air or in vacuo without acetone or ether is used the error is somewhat greater.

The source of this monosaccharid is not yet definitely known. The mold contains one or more polysaccharids, or perhaps a hemicellulose, which gives an opalescent appearance to the aqueous extract, and which may be precipitated in an amorphous condition by the addition of alcohol. The red color obtained with iodin suggests the presence of glycogen. It is very probable that an enzym acts on one of these carbohydrates, liberating a monosaccharid.

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[a] Vandervelde, A. J. J. Ueber Diffusion von Enzymen durch Cellulosemembrane. Biochemische Zeitschrift, vol. 1, pp. 399–408. Berlin, 1906.

On allowing the acetone to stand after it had been used for extracting the mold by the Albert-Buchner method, needle-shaped crystals soon formed on the walls of the containing vessel. In order to determine the nature of this substance, the crystals were dissolved in water and allowed to crystallize again. On evaporating, a sweet sirup was obtained, which after several days crystallized in long needles. The crystals melted at 166° C., did not rotate the plane of polarized light, and did not reduce Fehling's solution before or after boiling with acid. These are all properties of mannite, a hexatomic alcohol which is known to occur in many of the higher fungi.

The purpose of the experiments which follow is not only to demonstrate the presence of the different carbohydrate-splitting enzyms, but also to show the influence of nutrition upon the relative amounts of the enzyms formed. With the exception of the paper by Katz (see page 27) on the regulatory formation of amylase, that of Pottevin (see page 25) on the formation of lactase in cultures upon lactose, and a few bare statements by Duclaux, very little is known about the influence of nutrition of molds upon the relative amounts of intracellular enzyms produced. This question of nutrition was studied especially in connection with the carbohydrate-splitting enzyms of *P. camemberti.*

In testing the influence of nutrition upon the relative activities of the intracellular enzyms the following procedure was adopted: A series of culture media was prepared consisting of the usual inorganic salts and 3 per cent of another carbohydrate in place of the sucrose. The media were sterilized and inoculated in the usual way with spores of *P. camemberti.* The flasks were all kept under the same conditions of temperature and light, and the organism was allowed to reach the same stage of development in each case, then the mycelium was removed and dehydrated by the usual method with acetone and ether. The preparations obtained in this way were used throughout the whole series of experiments, and the results are therefore strictly comparable. In order to determine the efficiency of the acetone-ether method as compared with simple dehydration of the mycelium in vacuo, a preparation made by the latter method was included in the series and will be designated as "dried mycelium." Preparations from mycelia grown upon the following carbohydrates were tested: Sucrose, maltose, lactose, glucose, starch paste, and inulin. In a series of test tubes there were placed 10 c. c. of a 2 per cent carbohydrate solution and 0.1 gram mold powder from the different sources indicated. Toluene was used for an antiseptic, and the mixture was kept in the thermostat for a given length of time. Then the enzym action was arrested by plunging the tubes into boiling water. After cooling, the solutions were clarified by shaking with a definite volume of alumina cream and filtering. The rotation of the resulting solution was determined in a Schmidt and Haensch triple-field polariscope, using a 100 mm. tube. The readings are expressed in degrees Ventzke. Assuming that the change in rotation is proportional to the change in specific rotation when the carbohydrate is hydrolyzed by acid, the percentage of hydrolysis obtained can be calculated roughly. Although this method of calculation may not be strictly accurate, it gives approximate values which may be used for comparison.

Having briefly outlined the methods, we now turn our attention to the data obtained by their application. The enzyms will be discussed in the same order that was followed in the historical review. As some of the rarer carbohydrates, such as trehalose, melizitose, and gentianose, could not be procured, the observations are confined to the more commonly occurring carbohydrates.

AMYLASE.

Although *P. camemberti* will grow upon media containing various forms of starch as the source of carbon, the growth is not as rapid as that obtained with the simple monosaccharids and disaccharids. At the outset the germination of the spores is somewhat delayed, but later on the organism seems to adjust itself to the medium, and finally a dense mycelium is formed and normal fruiting bodies appear. Starting with the same amount of carbohydrate in the medium, soluble starch required several days longer for its complete disappearance than did sucrose. In nine days the medium no longer gave a color with iodin, but the achroodextrins persisted four or five days longer. In the case of starch paste the hydrolysis was still slower. At the end of ten days the upper layer of the medium was clear and gave no iodin reaction, but below this there was a layer of unchanged starch. Achroodextrin is much more readily utilized, and the delay in the germination of the spores is less pronounced.

As might be expected in view of the "regulatory formation" of amylase discovered by Katz in another species of *Penicillium*, *P. camemberti* when grown upon sucrose does not produce any great amount of amylase. When a 1 per cent paste of arrowroot starch was treated with 2 per cent of its weight of mold powder from the culture grown on sucrose, a blue color was still obtained with iodin after the mixture had been in the thermostat for a week. The failure to digest starch paste could not have been due to a destruction of amylase by the acetone-ether treatment, for a similar test with a fresh culture that had been dried directly gave results that were practically negative. In this case, however, the iodin reaction at the end of a week was somewhat purplish, showing that a portion of the starch must have been converted as far as the erythrodextrin stage.

The amylolytic activity of the enzym preparation was more pronounced when soluble starch was used in place of starch paste. Twenty cubic centimeters of a 1 per cent solution of soluble starch when digested with 0.1 gram of the powder (from sucrose) for three days gave 0.0685 gram cuprous oxid. Deducting 0.0060 for the blank, it will be seen that the amount of reducing sugar formed, calculated as dextrose, is 0.0286 gram, or nearly 13 per cent of the theoretical yield. At the end of a week no iodin reaction was obtained. The exact time required for the starch to reach the achromic point was, however, not determined in this case. The reducing sugar was calculated as dextrose for the reason that it yielded an insoluble osazone on treatment with the phenylhydrazin reagent, and this osazone was further identified by its crystalline form as phenylglucoseazone. The maltose, which is no doubt formed as an intermediate product, is evidently quickly decomposed into dextrose by another enzym.

The activity of the amylase is extremely small compared to that of malt or saliva. The highest dilution at which the enzym could still be demonstrated was determined in the following way: A 2 per cent solution of soluble starch was prepared and 10 c. c. placed in a series of test tubes. An extract of the mold powder was also prepared by extracting a gram of the powder for two hours with thirty times its weight of water. Different amounts of this extract were added to the starch solution and the total volume made up to 15 c. c. From time to time the progress of the digestion was determined by testing a portion of the solution with iodin. The data are tabulated below.

Effect of various amounts of mold enzym on digestion of starch.

Mold extract.	Color.		
	3 days.	5 days.	7 days.
5 cubic centimeters	Red	No color	No color.
2 cubic centimeters	Violet	Red	Do.
1 cubic centimeter	Blue	Violet	Red.
0. 5 cubic centimeter	do	Blue	Violet.
0.2 cubic centimeter	do	do	Blue.
0.1 cubic centimeter	do	do	Do.

A distinct change in color was noticed in the tube where an extract representing 0.007 gram of the mold powder was added to 10 c. c. of the 2 per cent starch solution and allowed to digest for a week.

It has already been shown that the mold preparation acts much more readily upon soluble starch than upon starch paste. Dextrin is still more readily digested. A specimen of "C. P." dextrin was obtained, which gave no color with iodin and no reduction with Fehling's solution, and the action of the mold powder was tested upon it. In three days a suspension of 0.1 gram mold powder in 20 c. c. of a 1 per cent solution of this dextrin yielded 0.1895 gram cuprous oxid by Allihn's method. Calculated as dextrose this would represent a hydrolysis of nearly 40 per cent, or about three times as great as that obtained in the same length of time with soluble starch.

Glycogen, on the other hand, is much less readily attacked than soluble starch or even starch paste. Two-tenths of a gram of glycogen under the same conditions yielded only 0.0019 gram cuprous oxid. There was apparently no diminution in the opalescence of the solution. On repeating the experiment and testing with phenylhydrazin no separation of osazone could be obtained.

The above experiments were all conducted with the preparation of mold that had been obtained from cultures on cane sugar. To determine the influence of nutrition on the formation of amylase the method already outlined was followed. Ten cubic centimeters of a 2 per cent solution of soluble starch were placed in each of a series of tubes, 0.1 gram of the mold from the different sources was added, and the mixture was left in the thermostat for forty-eight hours. An equal volume of alcohol was then added to precipitate the unchanged starch, the precipitation being facilitated by the addition of a little sodium chlorid. After filtering, the optical rotation was determined. The solution was then evaporated to remove the alcohol, and the residue taken up in a small volume of water. The iodin test was applied and also the reduction of Fehling's solution.

Test to determine the influence of nutrition on the formation of amylase.

Source of enzym.	Rotation.	Color with Iodin.	Fehling's test.
	Ventzke degrees.		
Sucrose	4.8	Red	+
Sucrose(dried mycelium)	5.0	Violet	+
Maltose	4.2	No color	++
Lactose	3.8	Red	+
Glucose	4.6	do	+
Starch	4.8	No color	++
Inulin	3.9	Violet	+
Blank	.4	Faint blue	–

The rotation in this case does not show the relative amount of hydrolysis. This is because the amount of dextrin not precipitated by alcohol at first increases, giving an increase in rotation, then the dextrin undergoes hydrolysis and the rotation decreases. It is therefore impossible to determine from the rotation alone whether the latter is increasing or decreasing. But the rotation and the iodin test together afford a satisfactory method of comparison. The mold obtained from the cultures on maltose, and more especially on starch, show the greatest amylolytic activity. In both of these cases the achromic point has been reached, and the reduction of Fehling's solution is stronger than that obtained with the other preparations.

In general it may be said that the amylolytic power of the mycelium of *P. camemberti* is rather feeble when the organism is cultivated upon cane sugar. Soluble starch is more readily digested than starch paste, and achroodextrin still more readily, while glycogen is only very slightly hydrolyzed. A more active preparation of amylase may be obtained by introducing starch or maltose into the culture medium in place of the cane sugar.

INULASE.

The enzym powder prepared from cultures of *P. camemberti* on sucrose had very little action on inulin. When inulin solutions were treated with a suspension of this powder for several days, then freed from the unchanged inulin by alcohol and tested with the phenylhydrazin reagent, only a very slight separation of osazone resulted. This was regarded as evidence of digestion, however, as no separation of osazone could be obtained in the control. Several repetitions of this test gave similar results. Quantitative determinations confirmed the evidence obtained by the phenylhydrazin test. One-tenth of a gram of the mold powder was suspended in 20 c. c. of a 1 per cent solution of inulin, and the mixture allowed to digest for three days. The amount of cuprous oxid then obtained by Allihn's method, after deducting the blank, was 0.0342 gram. This represents 0.0149 gram levulose, or a hydrolysis of about 7 per cent.

The variations in the amount of inulase that might be caused by differences in nutrition were determined in much the same way as in the preceding studies with amylase. Ten cubic centimeters of a 2 per cent inulin solution were placed in a series of tubes, and 0.1 gram of enzym powder from the various sources was added. After forty-two hours the digestion was stopped and the solutions clarified by shaking with 5 c. c. alumina cream. Polarimetric determinations were made upon the filtrate, with the following results:

The influence of nutrition on the formation of inulase.

Source of enzym.	Rotation.	Approximate hydrolysis.
	Ventzke degrees.	*Per cent.*
Sucrose	−2.6	3
Sucrose (dried mycelium)	−2.8	8
Maltose	−2.6	3
Lactose	−2.6	3
Starch	−2.6	3
Glucose	−2.6	3
Inulin	−5.9	92
Blank	−2.5	0

Here is a striking instance of the influence of nutrition upon enzym formation. All of the preparations showed some slight activity. But the mold that had been cultivated on inulin as the only source of carbon contained many times as much inulase as those which had been grown on other carbohydrates. The organism evidently adapts itself to the medium by increasing the amount of the particular enzym needed.

Inulin as a source of carbon for cultures of *P. camemberti* gives results similar to those obtained with starch paste, already mentioned. The germination is much delayed, and at first only a scanty growth of the organism is obtained. The mold then seems to thrive after it once gets a start, and produces normal fructification, though the mycelium is not as dense as that obtained with cane sugar or maltose. The complete inversion of the inulin requires more than two weeks, and the levulose is consumed nearly as fast as it is liberated.

In studying the rate of hydrolysis of inulin by the enzym, the preparation obtained from the culture on inulin was used, as the others possessed so little activity toward inulin. One gram of the powder was extracted in the usual way with thirty times its weight of water, and different amounts of the extract were added to 10 c. c. of a 2 per cent solution of inulin. The total volume was made up to 15 c. c. After forty-six hours in the thermostat the solutions were clarified with 5 c. c. alumina cream and the rotation was determined.

The hydrolysis of inulin with various amounts of the enzym.

Mold extract.	Rotation.	Approximate hydrolysis.
	Ventzke degrees.	*Per cent.*
5 cubic centimeters	−4.6	81
2 cubic centimeters	−3.6	48
1 cubic centimeter	−3.0	29
0.5 cubic centimeter	−2.6	16
0.2 cubic centimeter	−2.2	3
0.1 cubic centimeter	−2.1	....
0 cubic centimeter	−2.1	0

The amount of hydrolysis obtained in a given length of time increases therefore with increase in concentration of the enzym. As small an amount as 0.2 c. c. of a 3 per cent extract of the mycelium which had been grown on inulin gives an appreciable hydrolysis with 0.2 gram inulin dissolved in 15 c. c. water.

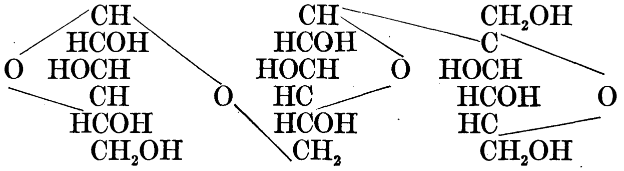
RAFFINASE.

In testing for raffinase the same procedure was followed as described in the preceding experiments. Twenty-four hours' digestion of raffinose with a suspension of the mold powder yielded reducing sugars which gave a copious precipitate of insoluble osazone. Sugar determinations were made as before, after allowing 20 c. c. of a 1 per cent raffinose solution to digest for three days with 0.1 gram mold powder. The figures are as follows: Digestion, gram Cu_2O, 0.2438; control, 0.0016; net increase, 0.2361; mold alone, 0.0061. This would represent in terms of monosaccharid a hydrolysis of about 50 per cent.

Varying amounts of a 3 per cent mold extract were added to 10 c. c. of a 2 per cent raffinose solution and the volume was made up to 15 c. c. The digestion was carried on for forty-two hours and the solutions were clarified in the usual way. The polariscope readings were as follows:

Mold extract.	Rotation.
	Ventzke degrees.
5 cubic centimeters	4.6
2 cubic centimeters	5.2
1 cubic centimeter	5.5
0.5 cubic centimeter	5.7
0.2 cubic centimeter	5.8
0.1 cubic centimeter	5.8
0 cubic centimeter	5.8

In the complex system resulting from the incomplete hydrolysis of raffinose, the percentage of hydrolysis can hardly be calculated from the rotation. The first stage in the hydrolysis is the liberation of levulose, then further hydrolysis would decompose the remaining disaccharid into dextrose and galactose, as indicated in the formula—



(Galactose.) (Dextrose.) (Levulose.)

The mold was found to grow readily upon raffinose as the source of carbon, the appearance of the colony being much the same as that obtained with sucrose. As a sufficient quantity of raffinose was not available for the preparation of mold powder from this source, the nutritional experiments were conducted with preparations from other sources.

The influence of nutrition on the formation of raffinase.

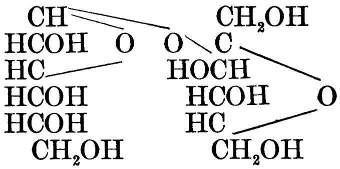
Sources of enzym.	Rotation.	Decrease.
	Ventzke degrees.	*Ventzke degrees.*
Sucrose	5.4	2.5
Sucrose (dried mycelium)	4.8	3.1
Maltose	7.0	0.9
Lactose	3.2	4.7
Starch	6.7	1.2
Glucose	6.8	1.1
Inulin	5.9	2.0
Blank	7.9	....

The decrease in rotation compared with the control shows that the raffinase was present in all of these preparations. The greatest activity was obtained with the mycelium that had grown on lactose, while that grown on sucrose was next in activity. With the other sources of sugar, the activity was considerably less. A significant fact is that both of the sugars which yielded the most raffinase contain two of the hexoses present in raffinose. The greater activity of the lactose preparation may be accounted for by the fact that the disaccharids containing galactose are relatively stable and the enzym necessary to decompose this intermediary melibiose is increased in amount by the adaptation of the mold to lactsoe, which is isomeric with melibiose. The relationship between lactase and melibiase is not known, but in view of the fact that lactose and methyl-d-galactosid are apparently hydrolyzed by the same enzym, there is some ground for supposing that this enzym may act on other galactosids as well.

SUCRASE.

Cane sugar is an excellent source of carbon for *P. camemberti*, as well as for other molds of the same and of related genera. Owing to the ease with which it can be procured and the readiness with which most saprophytic molds grow upon it, this sugar has formed the basis of most of the culture media described. Like other molds which utilize cane sugar, *P. camemberti* first inverts it. By following the hydrolysis of the sugar by means of determinations by Allihn's method it was found that the inversion was complete in six days, after 12 per cent of the sugar had been consumed. From that time on the sugar disappeared rapidly, until after twelve days no reduction could be obtained. Not until the ninth day was there any appreciable amount of sucrase in the medium; then the amount rapidly increased.

When cane sugar is hydrolyzed a molecule each of dextrose and levulose is formed. These two monosaccharids reduce Fehling's solution, while the original sucrose does not. The hydrolysis is also accompanied by a change in rotation which will be discussed presently. The splitting takes place as indicated in the structural formula—



Qualitative tests by the usual methods showed at once that sucrase is present in the mold preparation. When a 2 per cent solution of cane sugar was treated with 1 per cent its weight of mold powder and left in the thermostat for twenty-four hours, the resulting solution gave, on warming with the phenylhydrazin reagent, a copious separation of phenylglucosazone. It gave also a marked decrease in rotation and a strong reduction with Fehling's solution. All of these tests were negative in the control experiments.

Having demonstrated the presence of sucrase, some quantative determinations were then made. In this series of experiments, as well as in those following, a solution of rock candy was used, as it contained no trace of reducing sugar. Five cubic centimeters of a 2 per cent solution of this sugar were measured out into each of a series of tubes. A definite volume of mold extract, prepared by macerating the mold powder with thirty times its weight of water, and filtering, was then added and the total volume made up to 10 c. c. with distilled water. After forty-eight hours in the thermostat determinations were made by Allihn's method, the copper being weighed as cuprous oxid. As 5 c. c. of this extract yielded 0.0060 gram cuprous oxid without the addition of sucrose, the error was calculated in each case and deducted before estimating the percentage of hydrolysis. The results are set forth in the following table:

The hydrolysis of sucrose with various quantities of enzym.

Mold extract.	Cuprous oxid.	Invert sugar.	Hydrolysis.
	Gram.	*Gram.*	*Per cent.*
5 cubic centimeters	0.2168	0.0990	94.2
2 cubic centimeters	.2120	.0987	93.9
1 cubic centimeter	.2068	.0965	92.0
0.5 cubic centimeter	.1833	.0858	81.7
0.3 cubic centimeter	.1295	.0607	57.7
0.2 cubic centimeter	.0825	.0387	36.9
0.1 cubic centimeter	.0514	.0245	23.4
0.05 cubic centimeter	.0354	.0173	16.5
0 cubic centimeter	.0000		
5 cubic centimeters enzym alone	.0060		
Acid hydrolysis	.2229	.1051	100.0

The rate of hydrolysis, except at the upper limits, follows the law of Henri.

Similar results were obtained by the use of the polariscope. While the specific rotation of sucrose is 66.5° Ventzke, the dextrose and levulose formed have specific rotations of 52.7° and −93°, respectively, and the rotation of the equimolecular mixture resulting from hydrolysis would therefore be −20.1°. As the rotation in the control experiment is 3.6° Ventzke, the calculated rotation after complete inversion would be −1.1° Ventzke. From this the percentage of hydrolysis is estimated in each case. In this experiment varying amounts of a 3 per cent extract of mold (grown on sucrose) were added to 10 c. c. of a 2 per cent sucrose solution and the total volume was made up to 15 c. c. The digestion was carried on for twenty-four hours, then the solutions were clarified with 5 c. c. alumina cream and the optical rotation was determined as usual.

Mold extract.	Rotation.	Hydrolysis.
	Ventzke degrees.	*Per cent.*
5 cubic centimeters	−1.1	100
2 cubic centimeters	.0	77
1 cubic centimeter	1.5	45
0.5 cubic centimeter	2.4	25
0.2 cubic centimeter	3.2	9
0.1 cubic centimeter	3.4	4
0 cubic centimeter	3.6	0

To determine whether *P. camemberti* produces sucrase when other carbohydrates than sucrose have served as the source of carbon, mold preparations from different sources were used exactly as described under inulase and raffinase, and the rotation was measured after twenty-four hours.

Production of sucrase with various carbohydrates.

Sources of enzym.	Rotation.	Hydrolysis.
	Ventzke degrees.	*Per cent.*
Sucrose	−1.0	100
Sucrose (dried mycelium)	−1.0	100
Maltose	− .4	86
Lactose	−1.0	100
Glucose	.1	74
Starch paste	.2	71
Inulin	.2	71
Blank	3.2	0

The rotation after complete hydrolysis in this experiment would be −1.0° Ventzke, and from this the percentages of hydrolysis are calculated. It is evident from the table that sucrose is formed no matter which of the carbohydrates has served as the source of carbon in the culture medium. Yet sucrase is known to be a specific enzym. The highest result was obtained with the mold that had been cultivated on sucrose and lactose. The differences are by no means as great as those obtained in the experiments with inulase.

MALTASE.

The presence of maltase in the mold powder was first ascertained qualitatively by the phenylhydrazin test. A 1 per cent solution of maltose was treated for twenty-four hours with one-twentieth its weight of mold powder and an abundant precipitate of phenylglucosazone obtained by the usual test. This indicates a fairly active maltase. A quantitative determination by Allihn's method established this fact beyond doubt. In this experiment 20 c. c. of a 1 per cent solution of maltose was treated for a week with 0.1 gram mold powder. At the end of that time the amount of cuprous oxid obtained was 0.3504 gram, while the control gave 0.2274 gram. As 0.2274 gram cuprous oxid represents 0.1779 gram anhydrous maltose and this on complete hydrolysis would yield 0.3805 gram cuprous oxid, the hydrolysis may be taken as about 90 per cent.

In determining the relative activity of different amounts of enzym, the mold that had been cultivated on maltose was used. The experiment was conducted as usual, the time of digestion in this case being twenty-nine hours. Ten cubic centimeters of a 2 per cent maltose solution were used, and diluted to 20 c. c. before polarizing. On complete hydrolysis of maltose the specific rotation decreases from 138° to 52.7 ° Ventzke. In the following experiment the maltose solution before inversion had a rotation of 7.1° Ventzke, and the rotation after complete hydrolysis would therefore be 2.7° Ventzke.

Mold extract.	Rotation.	Hydrolysis.
	Ventzke degrees.	*Per cent.*
5 cubic centimeters	4.6	56
2 cubic centimeters	5.2	43
1 cubic centimeter	6.0	20
0.5 cubic centimeter	6.6	9
0.2 cubic centimeter	7.0	2
0.1 cubic centimeter	7.1	....
0 cubic centimeter	7.1	....

As in the preceding experiments with other enzyms, the influence of nutrition on the formation of enzym was determined. The experiments were conducted as before, using a 2 per cent solution of maltose, and allowing the digestion to go on for forty-two hours. The following table shows the relation between the nutrient carbohydrate and the maltose-splitting enzym:

Effect of nutrition on the formation of maltase.

Source of enzym.	Rotation.	Hydrolysis.
	Ventzke degrees.	*Per cent.*
Sucrose	8.7	18
Sucrose (dried mycelium)	8.4	23
Maltose	6.8	50
Lactose	8.6	20
Starch	6.6	53
Glucose	7.0	47
Inulin	8.9	15
Blank	9.8	....
Calculated for complete hydrolysis	3.8	100

Maltase is evidently present in all of these preparations. The carbohydrates which favor its formation are starch and maltose.

LACTASE.

The action of the mold powder on lactose is very slight indeed. The phenylhydrazin test, which was first applied, showed no evidence at all of digestion. On treating a 1 per cent solution of lactose with one-twentieth its weight of mold powder, no separation of glucosazone could be obtained by the usual procedure. On the contrary, the solution on cooling yielded characteristic crystals of phenyllactosazone. Determinations by Allihn's method showed, however, a very slight increase in reducing power. Twenty cubic centimeters of a 1 per cent lactose solution were taken and 0.1 gram mold powder added. After three days in the thermostat the reducing power was determined. Two experiments in which lactose from different sources was used gave the following figures:

[Expressed in grams Cu_2O.]

	Lactose plus enzym.	Lactose alone.	Net increase.
First experiment	0.3054	0.2923	0.0071
Second experiment	.2979	.2852	.0067

Enzym alone 0.0060.

The hydrolysis is approximately 1 per cent.

The increase in rotation after inversion of lactose is so slight that a conversion of 1 per cent could not be demonstrated in this way. If a 2 per cent solution of lactose were used and the polarimetric readings made in the usual way, a change in rotation of 0.1° Ventzke would represent a hydrolysis of about 10 per cent. Obviously the error is too great for this method to be applicable. Polarimetric determinations with lactose and enzym from different sources showed no appreciable increase within the limit of error.

The organism was found to grow fairly well upon media containing lactose, and the mold powder used in some of the preceding experiments showed that the enzyms obtained from the lactose-fed mycelium were in most cases as active as those obtained from sucrose. The activity toward lactose, however, was found to be greater. An experiment was conducted in exactly the same manner as those described above, and the reducing power determined as before.

Lactose plus enzym, gram Cu_2O, 0.3251; lactose alone, 0.2883; net increase, 0.0308. Enzym alone, 0.0060.

The amount of lactase present in the mycelium grown on lactose is therefore about four times as great as that present in the mycelium cultivated on sucrose. The very small amount of lactase formed under these conditions is not surprising. This enzym is the least abundant of the three inverting enzyms in either the vegetable or the animal kingdom. While ordinary yeast inverts sucrose and maltose, it is without action on lactose. Repeated attempts to find lactase in certain animal tissues have also resulted in failure.

SUMMARY.

By using pure cultures of a definitely identified mold and cultivating it under known conditions, the writer has endeavored to study the nature of the intracellular enzyms produced and the conditions favoring their formation. For various reasons *Penicillium camemberti* was found to be especially well adapted for such studies.

When *P. camemberti* is cultivated on a medium containing sucrose as the only source of carbon and sodium nitrate as the only source of nitrogen, a considerable number of enzyms are produced. They are retained for the most part by the mycelium until the time of fructification. By treating the mycelium with acetone and ether a dry powder results, which contains the enzyms in such form that they retain their activity indefinitely.

The powder prepared in this way was found to contain the following enzyms: Erepsin, nuclease, amidase, lipase, emulsin, amylase, inulase, raffinase, sucrase, maltase, and lactase. The lipase, inulase, and lactase were, however, quite feeble when the organism was grown on the cane-sugar medium.

The protease behaves exactly like Cohnheim's erepsin, except that its activity is greatest in neutral media or media faintly acid with acid phosphates. It digests casein, gelatin, and proteoses (Witte peptone), yielding a large percentage of amino acids, but does not attack native proteins to any appreciable extent.

The formation of erepsin is independent of the presence of protein or protein derivatives in the culture medium. An active erepsin is obtained when the organism is cultivated on a solution containing cane sugar and sodium nitrate as the only source of carbon and nitrogen.

A nuclease, liberating purins and phosphoric acid from yeast nucleic acid, is obtained independently of the presence of nucleic acid or organic nitrogen compounds in the culture fluid.

An amidase, or perhaps a group of amidases, is present, which liberates ammonia from urea, asparagin, benzamid, and alanin.

An enzym decomposing hippuric acid into benzoic acid and glycocoll is present in a very active form.

The carbohydrate-splitting enzyms—amylase, inulase, raffinase, sucrase, maltase, and lactase—are formed, no matter which of the corresponding carbohydrates has served as the source of carbon in the culture medium. The amount of enzym, which in some cases is quite small, may be materially increased by cultivating the organism on the corresponding carbohydrate. This is particularly true of inulase, amylase, and lactase, which ordinarily occur only in very small amount.

There is no evidence that enzyms not normally formed by the organism in demonstrable quantities can be developed by special methods of nutrition. The influence of adding a particular substratum to the medium is, therefore, not to develop an entirely new enzym, but to stimulate the production of the corresponding enzym, which is normally formed under all conditions.

By extending the application of this principle of regulatory formation of enzym the author hopes to be able to obtain more active preparations of lipase, nuclease, amidase, and other enzyms.

ACKNOWLEDGMENT.

The writer takes pleasure in acknowledging his great indebtedness for many helpful suggestions and for constant support throughout this investigation to Prof. Lafayette B. Mendel, of Yale University, in whose laboratory a considerable portion of the work was conducted.

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